

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

RELTON *et al.*

Appl. No.: 10/587,714

Filed: June 4, 2007

For: **Treatment of Conditions Involving
Dopaminergic Neuronal
Degeneration Using Nogo Receptor
Antagonists**

Confirmation No.: 2306

Art Unit: 1646

Examiner: Sandra L. WEGERT

Atty. Docket: 2681.0450001/EJH/JBF

Declaration of Dr. Stephen M. Strittmatter Under 37 C.F.R. § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Stephen M. Strittmatter declare and state as follows:

1. I received my education at Harvard College (A.B.), Johns Hopkins Medical School (M.D., Ph.D.) and Massachusetts General Hospital (Neurology Residency and Research Fellowship). A copy of my curriculum vitae is attached as Exhibit E.

2. I am currently employed at Yale University School of Medicine, where I am a Vincent Coates Professor of Neurology and hold the position of Director, Cellular Neuroscience, Neurodegeneration and Repair. My work involves the study of neural repair and neurodegeneration.

3. I am a named inventor of the above-identified application, and I am familiar with the pending claims and the April 14, 2010 Office Action.

4. I have been told by attorneys for Yale University that the specification of a patent application describes the claimed invention while the claims establish the scope of the invention. I understand that claims 1, 3, 4, 6, 10-12, 19-22, 24, and 26-35 are directed to methods of promoting regeneration or survival of dopaminergic neurons in a mammal displaying signs or symptoms of dopaminergic neuronal degeneration by administering directly to the CNS a therapeutically effective amount of a soluble form of a mammalian NgR1 or of an antibody or antigen-binding fragment thereof that binds to a murine or human NgR1. I understand that claims 23 and 36 are directed to methods of treating Parkinson's disease by administering directly to the CNS of a mammal a therapeutically effective amount of a soluble form of a mammalian NgR1 or of an antibody or antigen-binding fragment thereof that binds to a murine or human NgR1. I understand that the claims have been rejected in the Office Action for, among other things, lack of enablement. I have been told by attorneys for Yale University that a specification is "enabled" if, following the specification, a person having ordinary skill in the art could make and use the claimed invention using only routine experimentation. In my view, a "person of ordinary skill in the art" with respect to the above-identified patent application would be a person having at least post-doctoral level training and experience in the field of molecular biology and neurobiology and/or training as a specialist physician in neurology.

5. I understand that the Examiner alleges that the claims of the application encompass all methods of promoting restoration of dopamine neurons after any injury in the brain involving dopamine neurons, including those involved in diseases such as Parkinson's disease, using non-confirmed antagonists of NgR besides sNgR1. I also

understand that the Examiner alleges that the 6-hydroxydopamine (6-OHDA) model described and used in the application in rats and Nogo receptor knockout mice is not a valid model for Parkinson's disease or any of the diseases associated with dopaminergic neuronal degeneration encompassed by the claims.

6. On the contrary, based on my understanding of the field of neuroscience, it is my expert opinion that upon reading the current patent specification, one of ordinary skill would understand that the 6-OHDA model is a widely accepted and used model of Parkinson's diseases and other diseases associated with dopaminergic neuronal degeneration. In addition, the 6-OHDA model is predictive of success in protecting degeneration of dopaminergic neurons.

The claims encompass methods of promoting regeneration or survival of dopaminergic neurons in a mammal displaying signs or symptoms of dopaminergic neuronal degeneration. Examples of such conditions include Parkinson's disease, multiple system atrophy, striatonigral degeneration, olivopontocerebellar atrophy, Shy-Drager syndrome, motor neuron disease with parkinsonian features, Lewy body dementia, progressive supranuclear palsy, cortical-basal ganglionic degeneration, frontotemporal dementia, Alzheimer's disease with parkinsonism, Wilson disease, Hallervorden-Spatz disease, Chediak-Hagashi disease, SCA-3 spinocerebellar ataxia, X-linked dystonia-parkinsonism (DYT3), Huntington's disease (Westphal variant), prion disease, vascular parkinsonism, cerebral palsy, repeated head trauma, postencephalitic parkinsonism and neurosyphilis. While these conditions have different etiologies, they are all characterized by degeneration of dopaminergic neurons.

The 6-OHDA model of dopaminergic neuronal degeneration was developed in the 1960's as a tool to lesion the nigro-neostriatal dopamine system. Since then, 6-OHDA lesioning in rodents has been used extensively as a model for diseases affected by dopaminergic neuronal degeneration. Thousands of papers have been published that report on the use of the 6-OHDA model to study the effects of and to identify molecules that can be used to promote the regeneration or survival of dopaminergic neurons. *See, e.g.*, Fuxe and Ungerstedt, *Pharmac. Ther. B* 2:41-47 (1976) (Exhibit A); Tolwani, R.J., *et al.*, *Lab. Animal Sci.* 49:363-71 (1999) (Exhibit B); Betarbet, R., *et al.*, *BioEssays* 24:308-18 (2002) (Exhibit C); Deumens, R., *et al.*, *Exp. Neurology* 175:303-17 (2002) (Exhibit D) (and the references cited therein). And although other models of dopaminergic neuronal degeneration exist, these models are either no better at predicting efficacy or are worse.

For example, in Parkinson's disease, which is a sporadic and idiopathic disease that is still not very well understood, other toxin-induced models and genetic models have been developed. *See* Tolwani, R.J., *et al.*, *Lab. Animal Sci.* 49:363-71 (1999) (Exhibit B); Betarbet, R., *et al.*, *BioEssays* 24:308-18 (2002) (Exhibit C). Besides 6-OHDA, the only other model that is used actively and is similarly robust is the toxin-induced model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). However, while MPTP is an alternative to the 6-OHDA model, other toxin-induced models, such as rotenone, are less robust and the genetic models that have been developed are simply not as good. *See, e.g.*, Betarbet, p. 312, Table 1 (Exhibit C).

The 6-OHDA model in rats with unilateral lesioning has been the most commonly used animal model of Parkinson's disease and has been instrumental in its

contributions to preclinical research of the disease. *See* Deumens, R., *et al.*, *Exp. Neurology* 175:303-17 (2002) (Exhibit D). 6-OHDA became such a widely used model of Parkinson's disease because, in many aspects, it mimics the pathology and characteristic symptoms of the disease. *See* Deumens, p. 310, col. 1 (Exhibit D). For example, as with idiopathic Parkinson's disease, 6-OHDA administration can specifically destroy dopaminergic neurons in the substantia nigra of the midbrain, resulting in reduced levels of dopamine. Furthermore, some of the physical symptoms of Parkinson's disease, such as akinesia are induced by 6-OHDA lesioning. As mentioned above, while there are other models of Parkinson's disease that have been developed, no single model mimics all of the symptoms and pathology of Parkinson's disease.

In addition, the ability to quantify *in vivo* action of 6-OHDA by monitoring animal behavior has been invaluable to screening for therapeutics that protect dopaminergic neurons in Parkinson's disease and other diseases affected by dopaminergic neuronal degeneration. *See* Deumens, p. 306, Table 1 (Exhibit D). For example, following lesioning of an animal with 6-OHDA, specific behaviors such as animal rotation can be monitored and quantified when the animal is challenged with agents that target dopaminergic neurons. *See* Deumens, pp. 305-306, Figure 1, and Table 1 (Exhibit D); Fuxe and Ungerstedt, p. 41 (Exhibit A). This unilateral lesioning and rotation model was used in the present application in both rats and mice to demonstrate that blockade of Nogo receptor protects dopaminergic neurons from degeneration caused by 6-OHDA. Significantly, as shown in Example 1 of the application, treatment with the soluble Nogo receptor-1 construct in the 6-OHDA

lesioned rat increased dopaminergic neuronal survival in the substantia nigra, as well as levels of dopamine in the lesioned striatum.

Therefore, based on the findings that blockade of Nogo receptor protects dopaminergic neurons in the 6-OHDA model of dopaminergic neuronal degeneration, one of ordinary skill in the art would readily understand that Nogo receptor-1 antagonists could be used in methods to promote regeneration or survival of dopaminergic neurons. Furthermore, the person of skill would recognize that the Nogo receptor-1 antagonists for use in the methods of the application, such as soluble polypeptide fragments or antibodies or antigen-binding fragments that bind thereto, would be applicable to the treatment of those diseases affected by dopaminergic neuronal degeneration, including Parkinson's disease.

7. I further declare that the above statements made of my own knowledge are true and the above statements based on information and belief obtained from the references and documents discussed are believed to be true. Additionally, I declare that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Title 18 United States Code Section 1001, and that willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Respectfully submitted,



Date: 5 August, 2010

(RELTON *et al.*, APPL. NO. 10/587,714)

EXHIBIT A

ANTIPARKINSONIAN DRUGS AND DOPAMINERGIC
NEOSTRIATAL MECHANISMS: STUDIES IN RATS
WITH UNILATERAL 6-HYDROXYDOPAMINE
(=6-OH-DA)-INDUCED DEGENERATION OF THE
NIGRO-NEOSTRIATAL DA PATHWAY AND
QUANTITATIVE RECORDING OF ROTATIONAL BEHAVIOUR

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1. INTRODUCTION

Ungerstedt has described the high degree of lesion specificity that is obtained when 6-OH-dopamine (6-OH-DA) is used as a tool to lesion the nigro-neostriatal dopamine (DA) system (Ungerstedt, 1968, 1971a). Evidence has also been given that the rotational behavior induced in these rats by, for example, psychoactive drugs is highly related to the degree of DA receptor activity in the neostriatum (Andén *et al.*, 1966; Ungerstedt, 1971b). Thanks to the construction of a 'rotometer' (Ungerstedt and Arbuthnott, 1970), this behavior can be quantified and can be used to evaluate the effects of drugs on neostriatal dopaminergic mechanisms. Ungerstedt (1971c) has provided evidence that a supersensitivity to DA and apomorphine develops in the denervated neostriatum. Treatment with apomorphine, a DA receptor stimulating agent (Ernst, 1967; Andén *et al.*, 1967), results in rotation of the animal towards the intact side (Ungerstedt, 1971c), since the denervated side is more stimulated by apomorphine than the intact side. A catecholamine(CA)-releasing agent such as amphetamine (Carlsson *et al.*, 1966), on the other hand, will always result in rotation of the animal towards the denervated side, since no DA stores are available for release on the denervated side. Thus, in this model it is possible to differentiate between DA releasing agents and DA receptor stimulating agents. Using this model, Ungerstedt (1971c) has been able to show that dopa probably acts by formation of DA which acts mainly on the supersensitive neostriatal DA receptors, since rotation towards the intact side is obtained.

In studies with the '6-OH-DA rotation model', together with amine turnover studies, new types of possible antiparkinsonian agents have been discovered such as *m*-tyrosine (Andén *et al.*, 1970; Ungerstedt *et al.*, 1972), piribedil 7-(2"-pyrimidyl)-4-piperonyl-piperazine, ET 495; Corradi *et al.*, 1971, 1972a), and ergot alkaloids (Corradi *et al.*, 1972b).

2. ACTION OF *m*-TYROSINE

In contrast to *l*-dopa, *m*-tyrosine alone caused no rotational behavior with the exception of a few animals and only when high doses of 100-300 mg/kg were used (see Fig. 1). *l*-Dopa alone is effective in doses down to 10 mg/kg (Ungerstedt, 1971c). However, in combination with a peripheral dopa decarboxylase inhibitor, benserazide, *N*-(*dl*-seryl)-*N*¹-2,3,4-trihydroxybenzylhydrazine (Burkard *et al.*, 1962; Pletscher and Gey, 1963; Porter *et al.*, 1962), *dl*-*m*-tyrosine and *l*-*m*-tyrosine caused a dose-dependent increase in rotational behavior towards the intact side, demonstrating that the denervated DA receptors have become overactive. The threshold dose was 10 mg/kg and the *l*-form seems to be the active form. The action of *m*-tyrosine is probably mediated via formation of *m*-tyramine by decarboxylase in the denervated neostriatum and subsequent stimulation of the supersensitive DA receptors by

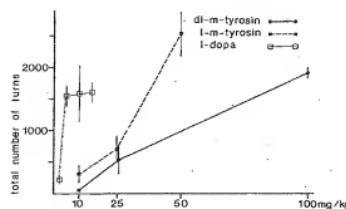


FIG. 1. The effect of *m*-tyrosine treatment on rotational behavior in rats with a 6-OH-DA-induced degeneration on the nigro-striatal DA pathway in combination with peripheral decarboxylase inhibition (benserazide), 50 mg/kg. A dose-dependent increase is observed. The *l*-form appears to be the active form. In the lower dose range *l*-dopa is about four to five times as active as *l*-*m*-tyrosine, but *l*-*m*-tyrosine does not reach a plateau value as rapidly as *l*-dopa. Means \pm SEM are given.

m-tyramine. The ethylester of *l*-*m*-tyrosine had a similar potency as *l*-*m*-tyrosine.

It is interesting to compare the effects of benserazide-*m*-tyrosine with those of benserazide-*l*-dopa treatment. The results show that *l*-dopa is five times as potent as *l*-*m*-tyrosine in this combined treatment with benserazide. In the higher dose-range it should be noted that whereas the animals treated with benserazide-*m*-tyrosine continue to show some increases in total numbers of rotations, the benserazide-dopa-treated rats have reached a plateau and do not show further increases in rotational behavior. Instead stereotyped licking and biting behavior develops.

The effects of *l*-*m*-tyrosine are potentiated by MAO inhibition and blocked by pretreatment with a DA receptor blocking agent such as spiroperidol or haloperidol. When the dose of benserazide is increased, the latency for onset of rotations is increased, supporting the view that *l*-*m*-tyramine is the active compound. Thus, when the dose of benserazide is increased, the cerebral decarboxylase is also inhibited. Similar results have been obtained with combined benserazide-dopa treatment (Ungerstedt, 1971c). With a dose of 50 mg/kg benserazide, which mainly inhibits peripheral decarboxylase, there was a difference in the latency for onset of *l*-dopa- and *l*-*m*-tyrosine-induced rotations. After *l*-dopa the rotations started after about 10–15 min, whereas after *l*-*m*-tyrosine the rotations started after about 30–40 min. This difference may be related to a slower decarboxylation of *m*-tyrosine and/or to the lower capacity of *m*-tyramine to activate the DA receptors.

The effects of *m*-tyrosine have also been tested in another parkinsonian-like model, i.e. on its action on tremor in monkeys with a ventromedial tegmental lesion (Goldstein *et al.*, 1969). Like *l*-dopa, *l*-*m*-tyrosine alone was found to induce a relief of tremor in a dose of 15 mg/kg. The effects lasted for about 15–30 min and full tremor was restored after about 2 hr (see Ungerstedt *et al.*, 1972). *l*-Dopa in this model was only about twice as active as *l*-*m*-tyrosine, and *l*-*m*-tyrosine was active in the absence of peripheral decarboxylase inhibition in contrast to what was found in rats. Thus, it seems reasonable that *l*-*m*-tyrosine could be an important new drug in the treatment of Parkinson's diseases via formation of the false transmitter *l*-*m*-tyramine in the neostriatum. *m*-Tyrosine, unlike dopa, is not metabolized by COMT but only by MAO, which may be an advantage, since *m*-tyrosine should not effect the levels of the methyl donor, *s*-adenosylmethionine, in the brain. Furthermore, the milder DA receptor stimulation induced by *m*-tyramine may be another advantage, since hyperkinesis seen after dopa treatment might be avoided. This is shown in the present experiments on rats in as much as stereotyped behavior in rats may be compared to hyperkinesis in man.

3. ACTION OF OTHER AMINO ACIDS

5-Hydroxytryptophan (5-HTP; 100–200 mg/kg, i.p.), α -methyldopa and α -methyl-*m*-tyrosine have not been found to induce any rotational behavior in rats with a unilateral

6-OH-DA-induced degeneration of the nigro-neostriatal DA system. These results indicate that the amines formed from these amino acids, 5-HT, α -methyl-DA and α -methyl-*m*-tyramine respectively, are not capable of stimulating the DA receptors nor of releasing DA in the neostriatum. These results thus demonstrate the specificity of the DA receptors. The addition of an α -methyl group obviously abolishes the DA receptor-stimulating property of *m*-tyramine (Andén *et al.*, 1970; Ungerstedt *et al.*, 1972). The effects of 3-*O*-methyldopa have also been studied, since this compound is one of the major metabolites formed in patients treated with dopa (Pletscher *et al.*, 1967; Tissot *et al.*, 1969) and then slowly demethylated to dopa (Bartholini *et al.*, 1971; Davidson *et al.*, 1971). It has therefore been postulated that 3-*O*-methyldopa might be effective in the treatment of Parkinson's disease, since in this way a continuous formation of dopa could be obtained from an endogenous depot and therapeutic effects have in fact been reported (Gauthier *et al.*, 1971). However, in our studies large and repeated doses of 3-*O*-methyldopa (100–500 mg/kg) have not induced any rotational behavior in the rats. These results rather suggest that the dopa formed from 3-*O*-methyldopa is too small to cause sufficient stimulation of the DA receptors. Therefore, of the amino acids tested, so far only *m*-tyrosine may be of value in the treatment of Parkinson's disease.

4. ACTION OF DA RECEPTOR-STIMULATING AGENTS

Apomorphine is a well-known drug causing a potent but short-lasting stimulation of DA receptors (Ernst, 1967; Andén *et al.*, 1967). Ungerstedt (1971c) has shown that this drug induces marked rotation towards the intact side. These results are explained by the development of supersensitivity to DA or DA receptor-stimulating agents in the denervated neostriatum. Because of the short-lasting effect of apomorphine and the fact that marked stereotyped biting behavior was induced in higher doses, it will be difficult to use apomorphine as a tool in treatment of parkinsonian patients, although some beneficial effects have been reported (Düby *et al.*, 1971). We have therefore tested a large number of apomorphine derivatives in order to find DA receptor-stimulating agents with a prolonged action. However, most of the apomorphine derivatives were inactive in our rotometer model. Only 7- and 11-hydroxy-noraporphines were weakly active (Granchelli *et al.*, 1971). Therefore we have tested other compounds in this model and in our amine turnover model. It is known that drugs stimulating monoamine receptors will cause a compensatory reduction in amine turnover.

In an attempt to find other DA receptor-stimulating agents we have tested various drugs for their ability to change DA turnover or induce rotational behavior. In these studies we have found two new types of DA receptor-stimulating agent, i.e. piribedil (ET 495; Corrodi *et al.*, 1971, 1972a) and ergot alkaloids (ergocornine and 2-brom- α -ergocryptine; Corrodi *et al.*, 1972b). Piribedil was found to cause a potent and prolonged stimulation of DA receptors as evidenced by marked rotational behavior towards the intact side and a marked reduction of DA turnover (Corrodi *et al.*, 1971, 1972a; see Fig. 2a, b). It is true that apomorphine is about four times as active as piribedil in the lowest dose range. However, it is important to note that in the higher dose range the total number of rotations seen after piribedil will continue to increase, whereas the apomorphine-induced rotational behavior reaches a plateau value due to induction of marked stereotyped licking and biting activity. With doses of above 50 mg/kg of piribedil, the rats could rotate for several days, demonstrating the prolonged effect of piribedil. More clinical trials with piribedil are therefore needed.

It is not known whether piribedil is active in itself or if an active metabolite is formed which is responsible for the DA receptor stimulation. The formation of an active metabolite is indicated by the fact that piribedil, in contrast to apomorphine, has little activity when given locally into the neostriatum. However, the formation of this metabolite must be very rapid, since rotational behavior is observed only a couple of minutes after systemic treatment with piribedil.

Another discovery in these studies was the observation that ergot alkaloids were

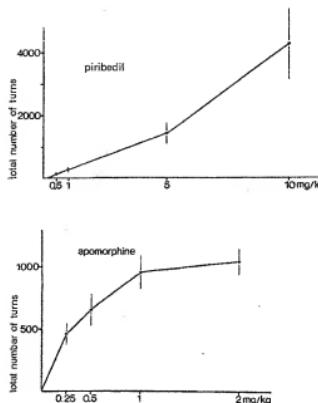


FIG. 2. The effect of apomorphine and piribedil on rotational behavior in rats with a 6-OH-DA-induced degeneration on the nigro-striatal DA pathway. Apomorphine and piribedil are observed to cause a dose-dependent increase in this behavior, the rats rotating to the intact side. Thus, dopamine receptors on the denervated side are stimulated more than those on the intact side. Apomorphine is in the lower dose range four to five times more potent than piribedil. However, with increasing dosage the animals treated with piribedil will continue to show increases in total number of turns, whereas the animals treated with apomorphine rapidly reach a plateau value. Means \pm SEM are given.

active in our tests, suggesting that these compounds can also stimulate neostriatal DA receptors. The threshold dose for inducing rotational behavior towards the intact side by ergocornine was 0.25 mg/kg and with 5 mg/kg marked rotational behavior was observed (see Fig. 3). A marked reduction of DA turnover was also present. 2-brom- α -ergocryptine was as active as ergocornine, and showed a very prolonged action. These results suggest that the testing of ergot alkaloid derivatives may be of help in developing new types of antiparkinsonian agents. It is obvious that these compounds have to be less toxic than ergocornine.

5. ACTION OF CATECHOLAMINE-RELEASING AGENTS

The effects of the catecholamine-releasing agent amphetamine have been studied extensively in the rotometer model (see Andén *et al.*, 1966; Ungerstedt, 1971b). Amphetamine induces a dose-dependent increase in rotational behavior towards the

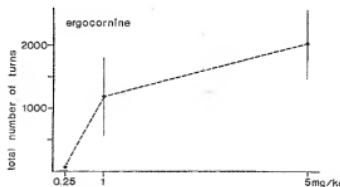


FIG. 3. The effect of ergocornine on rotational behavior in rats with a 6-OH-DA-induced degeneration on the nigro-striatal DA pathway. A dose-dependent increase in this behavior is observed, the rats rotating to the intact side. Thus, the dopamine receptor is stimulated more on the denervated side than on the intact side. Means \pm SEM are given.

denervated side. It was of particular interest to evaluate the effects of *d*-amphetamine compared to *dl*-amphetamine, since it has been postulated by Snyder and co-workers (Taylor and Snyder, 1971) that the differences between the actions of *d*- and *l*-forms of amphetamine mainly concern NE neurons and not DA neurons. However, Svensson (1971) has given evidence that the *d*- and *l*-forms are equipotent in releasing NE, whereas the *d*-form may be more active in releasing DA than the *l*-form as evaluated both functionally and chemically. Our results also indicate that *d*-amphetamine is more potent in releasing DA than the *l*-amphetamine (see Fig. 4). Furthermore, Taylor and Snyder (1971) have described that the *d*-form is twice as active as the *l*-form in eliciting stereotyped gnawing behavior. It is likely, therefore, that *d*-amphetamine, particularly from a theoretical point of view, could be a useful tool in the treatment of Parkinson's disease in view of its higher DA-releasing capacity. However, its use is excluded owing to its well-known addictive properties. This holds true for other drugs of this type such as phenmetrazine and methylphenidate, which also have been shown to induce rotational behavior towards the intact side and to release DA and NE. On the other

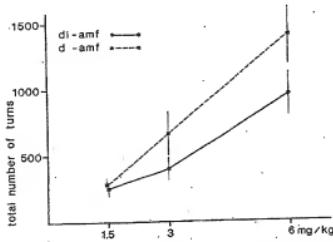


FIG. 4. The effect of amphetamine on rats with a 6-OH-DA-induced degeneration on the nigro-striatal DA pathway. A dose-dependent increase is observed towards the denervated side. Thus, dopamine receptors are stimulated more on the intact side than on the denervated side. The *d*-form is more active than the *l*-form. Means \pm SEM are given.

hand, weak CA-releasing agents probably lack narcotic effects. An example of a drug of this type is amantadine which in high doses (50–100 mg/kg) has been shown to cause rotation towards the denervated side (Farnebo *et al.*, 1971) and to release DA and NE (Strömberg *et al.*, 1970; Scatton *et al.*, 1970; Farnebo *et al.*, 1971). However, it is obvious that antiparkinsonian agents that act mainly by releasing DA can only be effective when some intact DA terminals still remain in the neostriatum. Otherwise, DA receptor-stimulating agents but not DA-releasing agents will be effective.

It has recently been suggested by Snyder and co-workers (Taylor and Snyder, 1969) that anticholinergic drugs might act by blocking the DA membrane pump in the DA nerve terminal. However, recent studies suggest (Farnebo *et al.*, 1970; Fuxe *et al.*, 1970) that the main action of anticholinergic drugs is via their known property to block cholinergic activity. However, as pointed out by Snyder and co-workers (Coyle and Snyder, 1969) and by our group, some anticholinergic drugs have a presynaptic action on the DA terminal, i.e. benzatropine and etybenzatropine have a capacity to block the uptake-concentration mechanism in the cell membrane of the DA nerve terminal. These types of anticholinergic drugs may be particularly useful in combination with dopa in the treatment of parkinsonian patients, since in addition to their anticholinergic effects they could directly potentiate the effect of dopa by increasing the leakage of DA from the nerve terminal onto the DA receptor.

In the rotometer model, it has been found that atropine and scopolamine cause rotation of the rat towards the denervated side. The mechanism for this effect is probably a blockade of the neostriatal cholinergic receptors antagonistic to the DA system, making possible an increased DA influence in the intact neostriatum. On the

denervated side, the blockade of the cholinergic pathway is probably of less importance, since the DA system has degenerated.

6. SUMMARY

The results of the present study illustrate the importance of using rotational behavior in rats with a unilateral 6-OH-DA-induced degeneration of the nigro-neostriatal DA system as an index of neostriatal DA receptor activity, particularly when combined with turnover analysis of DA. In this way it has been possible to develop new drugs that can be useful in the treatment of parkinsonian patients such as piribedil, ergot alkaloid derivatives and to obtain evidence that not only *dopa* but also *m-tyrosine* can be a powerful antiparkinsonian agent.

ACKNOWLEDGEMENTS

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EXHIBIT B

Special Topic Overview

Experimental Models of Parkinson's Disease: Insights from Many Models

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Abstract: Toxin-induced and genetic experimental models have been invaluable in investigating idiopathic Parkinson's disease (PD). The neurotoxins—reserpine, 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and methamphetamine—have been used to develop parkinsonian models in a wide variety of species. Both 6-OHDA and MPTP can replicate the neurochemical, morphologic, and behavioral changes seen in human disease. The unilateral 6-OHDA rat model is an excellent model for testing and determining modes of action of new pharmacologic compounds. The nonhuman primate MPTP-induced parkinsonian model has behavioral features that best approximate idiopathic PD. These induced and genetic models have been used to study the pathophysiology of the degenerating nigrostriatal system and to evaluate novel therapeutic strategies. Important differences within these models provide insights into various aspects of the dopaminergic phenotype and its role as a target in disease. These models provide an avenue to evaluate many anti-parkinsonian compounds, such as levodopa, which was first evaluated in an animal model and is the gold standard of parkinsonian treatment today.

Idiopathic Parkinson's disease (PD), first described in 1817 by James Parkinson (1), is a common neurodegenerative disorder leading to the onset of clinical features, including bradykinesia (slowness of movement), resting tremor, rigidity, and postural imbalance (2). The disease affects 1% of the population over the age of 55 years (3). Even though age is the only identifiable risk factor for the disease, early-onset cases do exist. The disease leads to progressive dysfunction and destruction of the mesencephalic dopaminergic neurons responsible for producing and transporting dopamine, via the nigrostriatal tract, to the striatum (4) (Figure 1). Dopamine in dopaminergic neurons is packaged into vesicles and delivered to the presynaptic membrane where it is released and binds with the dopamine receptors on the postsynaptic targets in the striatum. This loss of dopaminergic neurons leads to a profound deficit in the neurotransmitter dopamine in the striatum. Clinical signs of disease appear when striatal dopamine is reduced by 80% (2–4). Histologic changes indicate a loss of dopaminergic neurons principally in the substantia nigra pars compacta (SNpc), and to a lesser extent in the ventral tegmental area and retrorubral field (5). A pathologic hallmark of PD is the appearance of Lewy bodies, which are intracytoplasmic neuronal inclusions found in the SNpc, locus ceruleus, and nucleus basalis of Meynert (6).

Through use of animal models, the loss of striatal dopamine was identified as the principal feature of PD (7, 8). This finding led to the treatment of PD by use of levodopa

(dihydroxyphenylalanine or L-DOPA), which still remains the gold standard of treatment. Levodopa, able to cross the blood-brain barrier, is metabolized to dopamine by the enzyme DOPA decarboxylase (9). Administration of levodopa leads to enhanced amounts of striatal dopamine. Even though levodopa remains efficacious throughout the course of PD, its usefulness becomes confounded due to development of motor complications, including dyskinesia (spontaneous or uncontrolled movements), which often is severe and debilitating. It is essential that new therapeutics are found to treat PD and to better understand its etiopathogenesis. Experimental models provide an important avenue to achieve these goals.

Pharmacologic agents and neurotoxins have been used to develop experimental models in a wide variety of species. In addition, genetic models have been identified or engineered. The objective of these compounds is to specifically lesion the basal ganglia at the level of the dopaminergic neurons (SNpc), their terminals (striatum or CPu), or the line of communication between them (forebrain bundle) (Figure 1). All these models lead to depletion of dopamine and therefore mimic the neurochemical deficits seen in people with PD.

The objective of this review is to highlight the various experimental models and their application in shedding light on the mechanisms responsible for nigrostriatal cell death and depletion of dopamine.

Reserpine and α -methyl-p-tyrosine models: Systemic administration of reserpine, a pharmacologic compound causing a depletion of catecholamines in the brain, led to an akinetic (absence of movement) state in the rabbit (8). Administration of L-DOPA reversed the reserpine-induced akinetic syndrome, indicating that behavioral recovery was dependent on dopamine replacement (8). This similarity of

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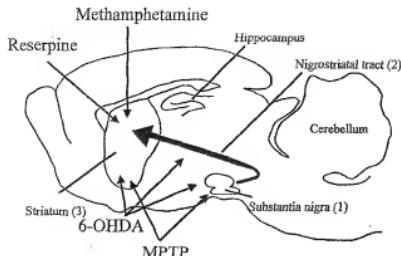


Figure 1. Neuroanatomic features of sites of lesioning of the rodent brain leading to dopamine depletion. The dopaminergic neurons of the substantia nigra project axons and their neurotransmitter dopamine (large filled arrow) to the striatum. The neurotoxic 6-hydroxydopamine (6-OHDA) is stereotactically injected into the substantia nigra and/or the ventral tegmental area (1), into the level of the nigrostriatal tract (forebrain bundle) (2) or into the striatum (3) to induce a lesion of the nigrostriatal system. Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or stereotactic injection of 1-methyl-4-phenyl-2,3-dihydroxyphenylidinium ion (MPP⁺) results in uptake of these compounds by the dopaminergic nerve terminal, causing degeneration of dopaminergic neurons of the substantia nigra. Systemic administration of methamphetamine results in degeneration of the dopaminergic nerve terminals in the striatum. Administration of reserpine leads to temporary dopamine depletion owing to release of intracellular dopamine pools in the striatum.

the akinetic syndrome in the rabbit to that in patients with PD led to the major breakthrough hypothesis that PD results from dopamine depletion. This hypothesis was confirmed with the subsequent observation that a dopamine deficiency develops in the striatum and substantia nigra in the brain of humans with PD (7).

The observation that signs of motor dysfunction associated with PD result from striatal dopamine depletion prompted application of reserpine in other species. For example, administration of reserpine to rodents induces a hypokinetic state (reduced movements) (10) due to depletion of dopamine at the nerve terminals. These motor changes are due to loss of dopamine storage capacity in intracellular vesicles (10). Similarly, systemic administration of α -methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, leads to similar clinical signs of disease (11). The motor deficits induced by these compounds are temporary and reversible in response to L-DOPA administration. Furthermore, these agents do not induce a morphologic change of the dopaminergic neurons.

These pharmacologically induced experimental models using reserpine have been used to investigate the therapeutic effects of many compounds, including dopamine replacement agents, such as L-DOPA, and dopamine receptor agonists (12). The therapeutic improvement of signs of motor dysfunction has yielded useful clinical information because these agents can be used as adjunct therapy for PD.

The 6-OHDA model: 6-Hydroxydopamine (6-OHDA) was the first agent discovered that had specific neurotoxic prop-

erties in the catecholaminergic nervous system (13–15). 6-OHDA uses the same catecholamine transport system as do dopamine and norepinephrine, leading to specific damage via oxidative stress to these neurons (14). To be neurotoxic to the brain, 6-OHDA must be administered by intracerebral or intraventricular injections because it is unable to cross the blood-brain barrier. The compound can be neurotoxic to the brain, however, if administered systemically in the neonatal animal, because the blood-brain barrier is not fully developed. Because all catecholaminergic neurons are affected by 6-OHDA, several strategies can be used to specifically target the dopaminergic system. The uptake of 6-OHDA into norepinephrine neurons can be blocked by systemic administration of des-methylimipramine (16, 17). Additionally, specificity is achieved by stereotactically targeting 6-OHDA to the substantia nigra, the ventral tegmental area, the nigrostriatal tract (forebrain bundle), or the striatum (18, 19).

Targeted injection leads to long-term destruction of dopaminergic neurons as early as 24 h after its administration (18, 20). The magnitude of the lesion is dependent on the amount of 6-OHDA applied, the site of injection, and inherent sensitivity between animal species. Loss of 80 to 95% of tyrosine hydroxylase immunoreactivity is achieved in most studies. Striatal dopamine values are depleted 2 to 3 days later (21), and this depletion may persist at least for 180 days (22, 23). Loss of dopaminergic neurons leads to loss of striatal dopamine content. These neurochemical observations correspond to behavioral changes (circling) that are observed when loss of striatal dopamine content exceeds 70% (24, 25).

The experimental parkinsonian model associated with 6-OHDA has been produced in many species, including mice, rats, cats, dogs, and nonhuman primates (26). The rat is most commonly used for the 6-OHDA parkinsonian model due to established stereotactic techniques and reasonable cost. Typically, only one hemisphere is injected, inducing a unilateral lesion leading to asymmetric motor behavior. Although unilateral injections lead to asymmetric motor behavior, bilateral injections in the rat can lead to hypokinetic, aphagic, adipsic behavior requiring nutritional support for survival of the animal (13).

The unilateral 6-OHDA model offers the advantage that the intact and lesioned nigrostriatal systems can be compared by observing the resulting motor behavior asymmetry. The asymmetric motor behavior associated with unilateral lesions results from a physiologic imbalance between lesioned and unlesioned sides. Spontaneous turning of the head or neck or circling behavior toward the lesion side may develop (Figure 2). The asymmetric behavior is usually short-lived (few days), but may persist, depending on severity of the lesion (27–29). Circling behavior is measured in a rotometer that may be computer integrated (25). The degree of circling can often be correlated with degree of lesioning (27, 30).

Not only can circling be spontaneous, but it may also be pharmacologically induced due to the changes in presynaptic and postsynaptic supersensitivity between the lesioned and the unlesioned sides (28, 29). Amphetamines, apomorphine, and L-DOPA are most commonly used to elicit circling behavior (Figure 2). Amphetamines and other dopamine

agonists act to increase the availability of endogenous dopamine by inducing dopamine release and inhibiting dopamine re-uptake (31). During the first week after lesioning, amphetamines induce release of dopamine from nonfunctional pools in the lesioned nigrostriatal system, resulting in contralateral (away from lesion) circling (Figure 2). After 1 week, amphetamines induce ipsilateral turning (toward the lesion) because dopamine pools in the lesioned side are depleted and dopamine release in the unlesioned side is increased. On the other hand, apomorphine, a dopamine receptor agonist, stimulates the receptors, which are up-regulated and hypersensitive on the lesioned side, leading to contralateral circling (27, 29, 30). Administration of L-DOPA, acting as a dopamine agonist stimulating the dopamine receptors, also leads to contralateral circling (29). Drug-induced circling behavior may differ from the aforementioned results and is influenced by many factors (32).

The 6-OHDA model falls short of duplicating idiopathic PD in humans. Pathologically, 6-OHDA does not affect as many regions of the brain, such as the locus ceruleus, as that associated with idiopathic PD. More importantly, the acute nature of the experimental model does not mimic the slow, progressive nature of dopaminergic neuronal degeneration (7). Some reports, however, suggest that degeneration may be progressive after 6-OHDA administration (20).

Despite some limitations, the 6-OHDA lesion model has been used to ascertain the efficacy and mechanism of action of anti-parkinsonian compounds. For example, the site of action of new drugs, whether they act pre- or postsynaptically, as well as whether they act as agonists or antagonists, can be determined (20). Additionally, this experimental model has been useful for evaluating the efficacy of transplantation and for testing neurotrophic factors, compounds that promote neuron survival (33, 34).

The MPTP model: In the early 1980s, several users of synthetic heroin developed acute parkinsonism. Investigation of the cause revealed the presence of the contaminating compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (35, 36). Intravenous injection of MPTP results in onset of clinical signs identical to those of idiopathic PD (35). Later, administration of MPTP to nonhuman primates indicated that MPTP selectively lesions the nigrostriatal system and induces behavioral changes remarkably similar to those of the human condition (37).

Presently, many of the biochemical details underlying the mechanism of action of MPTP have yet to be elucidated. It is known that MPTP is able to penetrate the blood-brain barrier where it is converted to 1-methyl-4-phenyl-2, 3-dihydroxydiphenylidium ion (MPP⁺) by the enzyme monoamine oxidase-B (MAO-B) localized in the glial and serotonergic neurons (38). Selective uptake of MPP⁺ into dopaminergic neurons via the dopamine transporter leads to its accumulation and accounts, at least in part, for its toxic specificity. Studies indicate that MPP⁺ acts on mitochondria by inhibiting production of ATP and by stimulating free radicals (39–41).

Administration of MPTP results in depletion of striatal dopamine and nigrostriatal cell death in a wide variety of animal species, including mice, cats, dogs, sheep, and nonhuman primates (Figure 3) (26). Even though almost all spe-

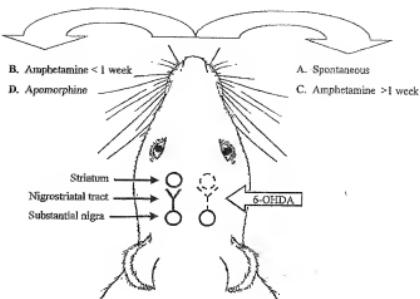


Figure 2. Circling behavior attributable to a unilateral lesion induced by use of 6-OHDA in the rat. A: Unilateral nigrostriatal lesion results in turning of head or circling toward the side of the lesion. B: Amphetamine administered during the first week after a lesion results in drug-induced circling behavior away from the side of the lesion. C: Amphetamine administered after the first week following a lesion results in circling behavior toward the side of the lesion. D: Apomorphine administered at low dosages results in circling away from the side of the lesion.

cies of animals develop some neurochemical or morphologic effects of MPTP, the degree of susceptibility among species varies on the basis of differences in sensitivity to MPTP (42). For example, the rhesus monkey (*Macaca mulatta*), an Old World nonhuman primate, is more sensitive than is the squirrel monkey (*Saimiri sciureus*), a New World nonhuman primate. Rats are essentially resistant to MPTP, whereas mice are somewhat sensitive (43). Different strains and sources of mice differ in sensitivity to MPTP. These differences are dependent on a number of factors, such as the intrinsic number of dopaminergic neurons (44, 45), and genetic differences reported to be influencing striatal MPP⁺ content (46). The C57BL/6 mouse strain is the most sensitive and most common MPTP rodent model used (47–49). Recent studies have confirmed that MPTP leads to dopaminergic cell death in C57BL/6 mice (47–49) (Figure 3). This degeneration of midbrain dopaminergic neurons develops in the substantia nigra and, to a lesser extent, the retrorubral field and ventral tegmental area, similar to sites of degeneration observed in humans with PD (45, 47–49).

The MPTP is typically administered to mice systemically, using a variety of regimens. The amount and route of administration corresponds to the extent of neurochemical and morphologic deficits. The selection of the route of administration (subcutaneous, intraperitoneal, intravenous, or intramuscular) and concentration of MPTP is principally based on the objective of the experimental question under investigation. For example, severe dopaminergic depletion and nigrostriatal cell death may be required to evaluate certain protective compounds, whereas a lesser degree of lesioning may be beneficial to investigate biochemical and morphologic changes in the course of recovery.

The behavioral effects of MPTP lesioning in mice are less marked than those seen in nonhuman primates (48, 50).

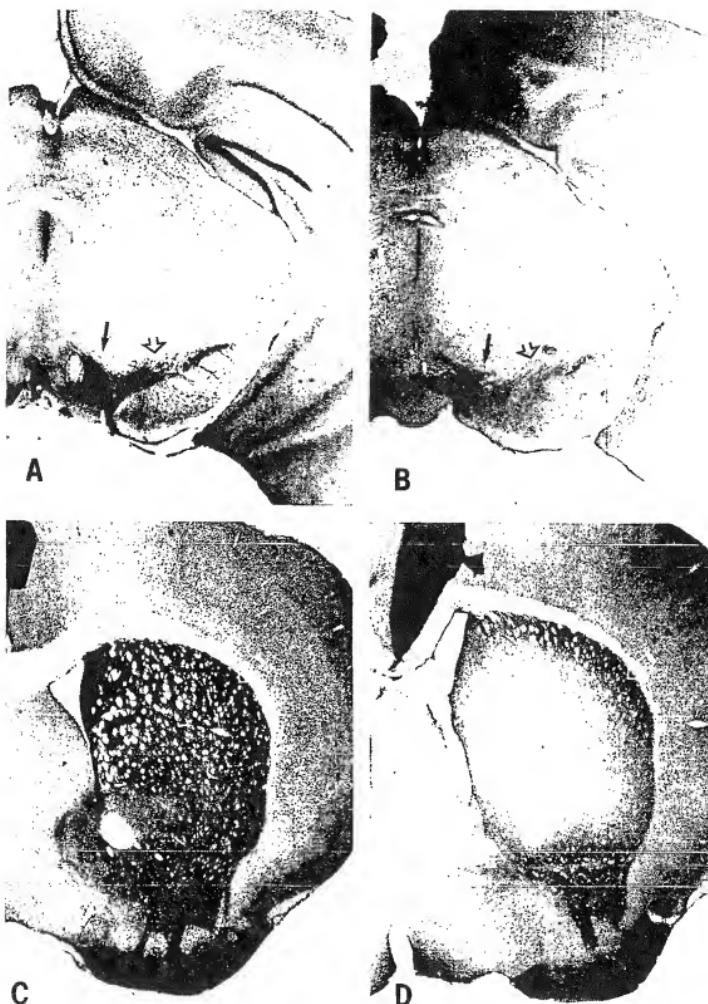


Figure 3: Photomicrograph of brain sections containing dopaminergic neurons after MPTP administration. (A) Untreated mouse brain, coronal section at the level of the mesencephalon, staining with antibody against the dopamine transporter (DAT). Open arrow indicates the intact substantia nigra pars compacta (SNpc), and closed arrow indicates the ventral tegmental area (VTA). (B) After MPTP exposure, many neurons in the SNpc are lost, with only a small reduction in VTA neurons. (C) Untreated mouse brain at the level of the midstriatum stained with an antibody against DAT. Strong immunoreactivity (dark) is seen throughout the striatum. (D) After MPTP exposure immunoreactivity is greatly reduced throughout the striatum.

Mice may develop initial short-term toxic effects, including hypersalivation, piloerection, and seizures (50, 51). Mice usually recover quickly and manifest normal spontaneous behavior within 24 h (51). Some short-term behavioral deficits, including hypokinesia (51) and decreased activity, have been reported (50, 52). Unilateral lesions, induced by stereotactic injection of MPTP into the nigrostriatal system, may induce circling behavior (53, 54).

The MPTP-lesioned nonhuman primate is probably the best model of human PD. This model develops pathologic and neurochemical changes similar to those of the human disease (43, 55-57). An important distinction, however, is the lack of progressive neuronal loss, as seen in idiopathic PD. Some studies have indicated, however, that administration of repeated low dosages of MPTP induces a progressive parkinsonism in the model (55, 58, 59). The MPTP-lesioned nonhuman primate model has provided an excellent tool to elucidate the structure and function of the basal ganglia as well as to study the mechanism of neurodegeneration, and to evaluate new therapeutics for PD, including surgery (transplantation, pallidotomy).

The behavioral changes observed in the MPTP-lesioned nonhuman primate best resemble those of the human disorder, a feature not seen in other species. These clinical features in the nonhuman primate include bradykinesia, rigidity, freezing (failure to initiate movement), balance impairment, and postural and/or resting tremor (60-62). Similar to its use in the human condition, administration of L-DOPA in the nonhuman primate reverses the behavioral signs. Continued administration of L-DOPA in the nonhuman primate leads to dyskinesias, which are involuntary and spontaneous motor movements consisting of chorea and/or dystonia, a feature seen in the late stages of idiopathic PD (63). Dyskinesia can lead to appreciable disability.

Variability exists within this model and is dependent on the regimen of MPTP administration and the species of nonhuman primate. For example, different species of nonhuman primates may require more frequent injections of MPTP to achieve the same degree of clinical parkinsonism and/or dopamine depletion (55). In addition, age is a consideration, because older animals tend to be more sensitive to MPTP than are younger animals (64). Systemic administration of MPTP leads to bilateral, often symmetric features of parkinsonism, with a corresponding partial loss of dopamine in the nigrostriatal system. In this systemic model, however, the degree of the lesion must be limited, because substantial bilateral dopamine depletion will compromise the animal's ability to maintain itself without therapeutic intervention. Additionally, dyskinesia is easily reproduced by use of pulsatile delivery of L-DOPA in this model (65). A cautionary note is that animals may tend to spontaneously recover; however, stable parkinsonism can be achieved (58, 62, 66-69). In the hemi-lesioned model, MPTP is administered into the internal carotid artery, leading to marked and almost complete unilateral destruction of the nigrostriatal system. This model features unilateral parkinsonism associated with hemi-neglect, corresponding to delayed reaction time on the contralateral side (70-72). Such animals also have spontaneous ipsilateral circling that can be reversed by adminis-

tration of L-DOPA. In this particular model, dopamine depletion unilaterally can be achieved to a greater degree, because the animal can maintain itself without therapeutic intervention due to the presence of an intact side.

The characteristics of each model become important when designing experimental drug studies. For example, a systemic model in which MPTP can be tailored to induce partial dopamine depletion may have greater usefulness when studying trophic factors, because residual neurons are still present and may therefore respond. Conversely, the hemi-lesioned model has usefulness in evaluating studies of dopamine replacement because unilateral complete loss of dopaminergic neurons can usually be achieved. Therefore, both models have provided important contributions toward understanding the mechanisms involved in nigrostriatal neurodegeneration and development of therapeutic interventions.

Methamphetamine. The administration of methamphetamine (METH) to rodents results in the long-term depletion of striatal dopamine and serotonin (73). The action of this toxin differs from that of MPTP in that dopamine is depleted at the level of dopaminergic terminals, not cell bodies. Under specific conditions, however, METH was reported to destroy cell bodies as well (74). Even though the mechanism of action is unclear, studies indicate that METH exerts its neurotoxic effects through the dopamine receptor and transporter because selective antagonists are able to block toxicity (75-77). It is well established that excitatory amino acids acting through the ionotropic glutamate receptors play a key role and that blocking these receptors is neuroprotective (78, 79).

Despite some gaps in the understanding of the mechanism of action of METH, this model has provided an important avenue to study the pharmacology and pathophysiology of the nigrostriatal system at the level of the dopaminergic terminals (80). Even though the major pathologic feature of PD is the death of nigrostriatal neurons, the loss of terminals in the time course of disease may also play an important role. The METH model provides an excellent tool to investigate the contribution of terminal function (through uptake of neurotoxins or trophic factors) and loss in the mechanism of disease.

Genetic models of PD: In addition to the experimental models developed using neurotoxic agents, several genetic rodent models have been discovered or engineered. The best-characterized spontaneous genetic mouse degenerative model is the weaver mouse. An autosomal recessive mutation in a potassium channel (81) leads to neuronal cell death in the cerebellum and the nigrostriatal system. In the midbrain, there is a significant reduction in tyrosine hydroxylase-positive neurons of the substantia nigra pars compacta and the retrorubral nucleus (82), with a corresponding reduction in striatal dopamine (83). These mice have been used principally for evaluating the efficacy of intrastriatal grafting of fetal dopaminergic cells (84). In addition to this mouse model, the mutant circling (ci) rat with abnormal and drug-induced circling behavior, similar to the 6-OHDA-induced circling model, has been reported (85). Furthermore, the AS/AGU rat, another natural rat mutant derived from the Albino Swiss (AS) rat strain, has motor changes, such as a staggering gait, hind limb rigidity, and difficulty in move-

Table 1. Neurotoxins and their effects

Neurotoxin	Species	Route of administration	Behavior	Systems affected	Pathologic features
Reserpine	Rodents/ rabbits	Systemic	Hypokinesia	Depletion of catecholamines	None
	Rat	Intracerebral or intraventricular	Behavior asymmetry (circling)	Dopaminergic and noradrenergic systems	Degeneration of dopaminergic neurons
MPTP	Mouse	Systemic	Usually none long term	Dopaminergic system	Degeneration of dopaminergic neurons in C57BL/6 strain
	Nonhuman primate	Systemic Unilateral carotid artery injection (hemislimed model)	Hypokinesia, rigidity, tremor Unilateral bradykinesia, tremor	Dopaminergic system Dopaminergic system	Degeneration of dopaminergic neurons Degeneration of dopaminergic neurons
Methamphetamine	Rodents	Systemic	Hyperactivity	Depletion of striatal dopamine and serotonin	Degeneration of dopaminergic nerve terminals

The most common species and routes of administration for the specific toxin are indicated. Behavior indicated is that most commonly and consistently seen.
6-OHDA = 6-hydroxydopamine; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

ment initiation. These behavioral changes result from a substantial loss of dopamine in the extracellular fluid of the striatum, suggesting a defect of dopaminergic neuron function (86). The weaver mouse, circling rat, and AS/AGU rat are excellent models for investigating nigrostriatal cell death and dopaminergic neuron function.

Information obtained from the neurochemical and neurotoxin models have specifically been applied to develop engineered models with alterations of the nigrostriatal system. Gene disruption by gene targeting has been used to generate mice deficient of specific dopaminergic phenotypes (87-89). Disruption of the dopamine D2 receptor gene, for example, leads to locomotor impairment in mice that is similar to that of PD (88). These mice have been useful in evaluating the role of dopamine receptors in synaptic plasticity (88). The recent identification of the missense mutation in the α -synuclein gene leading to early-onset familial PD will undoubtedly lead to development of transgenic mice with targeted disruption or overexpression of the mouse α -synuclein homolog (90). The advantages of these spontaneous and engineered genetic models include the ability to study progressive neurologic impairment, progressive neuronal death, and the adaptive mechanisms that compensate for dopamine depletion (91).

In our studies, genetic models (transgenic mice expressing neurotrophic factors) are being used to evaluate treatments for PD. This engineered mouse will allow investigation of the role of neurotrophic factors in protecting against neurotoxic injury of the dopaminergic system. Furthermore, this model allows study of the effects of neurotrophic factors on dopamine utilization, uptake, and release. Most importantly, however, this model system can be used to evaluate the effects of neurotrophic factors when they are provided after neuronal injury (as with MPTP) on recovery of the nigrostriatal system.

Conclusion

Experimental models have been invaluable in examining the pathophysiology of PD. The link between dopamine depletion and PD was first hypothesized as a result of studies in the reserpine-treated rabbit (6). Parkinsonian models have been developed in various species with use of 6-OHDA

and MPTP. Use of MPTP in the mouse leads to neurochemical deficits and morphologic lesion of the nigrostriatal system, and represents an excellent model for testing neuroprotective agents against MPTP lesioning. The unilateral 6-OHDA rat model is an excellent model for testing pharmacologic compounds and agents and distinguishing modes of their action. Administration of MPTP to the nonhuman primate leads to behavior deficits that most closely resemble the clinical features of idiopathic PD. Use of MPTP in the nonhuman primate also results in lesions of the basal ganglia similar to those of human PD. Studies of the dopaminergic nerve terminals have been carried out using the methamphetamine-induced models. Genetic models have been useful for studies on progressive nigrostriatal cell death and on specific aspects of the dopaminergic system.

These models have been instrumental in evaluating novel therapeutic interventions (92, 93). For example, the effectiveness of embryonic neural transplantation in the 6-OHDA rat has been documented (94, 95). Other studies have used animal models to study the efficacy of neurotrophic factors in treating PD. Using the MPTP and 6-OHDA parkinsonian models, encouraging results have been obtained after grafting of genetically modified cells expressing neurotrophic factors (96-99). Additionally, virus-mediated gene-delivery approaches have been used to deliver neurotrophic factors to parkinsonian models (100-103). Animal models, therefore, have substantially contributed to advancing our knowledge of PD.

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EXHIBIT C

Animal models of Parkinson's disease

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Summary

Animal models are important tools in experimental medical science to better understand pathogenesis of human diseases. Once developed, these models can be exploited to test therapeutic approaches for treating functional disturbances observed in the disease of interest. On the basis of experimental and clinical findings, Parkinson's disease (PD) was the first neurological disease to be modeled and, subsequently, to be treated by neurotransmitter replacement therapy. Agents that selectively disrupt or destroy catecholaminergic systems, such as reserpine, methamphetamine, 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine have been used to develop PD models. Recently, it has been found that agricultural chemicals, such as rotenone and paraquat, when administered systemically, can reproduce specific features of PD in rodents, apparently via oxidative damage. Transgenic animals that over-express α -synuclein are used to study the role of this protein in dopaminergic degeneration. This review critically discusses animal models of PD and compares them with characteristics of the human disease. *BioEssays* 24:308–318, 2002.

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Introduction

Parkinson's disease (PD), first described by James Parkinson in 1817, is a chronically progressive neurodegenerative disease, affecting at least 1% of the population over the age

of 55. Fully developed PD comprises motor symptoms such as tremor, rigidity (stiffness) and bradykinesia (slowness of movement). The sine qua non of PD is degeneration of the neural connection between the substantia nigra (SN) and the striatum,⁽¹⁾ two brain regions (nuclei) essential for normal motor function (Fig. 1). The striatum receives its dopaminergic input from neurons of substantia nigra pars compacta via this *nigrostriatal pathway*.⁽²⁾ Progressive degeneration of the nigrostriatal dopaminergic pathway results in profound striatal dopamine deficiency, and clinical signs of PD appear when striatal dopamine is reduced by about 80% (Fig. 2). Striatal dopamine deficiency—and the resultant changes in motor circuitry—are believed to underlie many of the clinical manifestations of PD.^(3–7)

Another important pathological feature of PD is the presence of filamentous, cytoplasmic inclusions called Lewy bodies. In PD, Lewy bodies are present in the dopaminergic neurons of SN, as well as in other brain regions including the cortex and magnocellular basal forebrain nuclei.⁽⁸⁾ A major component of the Lewy body is a protein called α -synuclein.⁽⁹⁾ Although mutations in the α -synuclein gene have been associated with rare familial cases of PD,⁽¹⁰⁾ α -synuclein is found in all Lewy bodies, even in the vast majority of PD cases without α -synuclein mutations.⁽⁹⁾ Thus, it may play a central role in disease pathogenesis. Furthermore, transgenic animal models have suggested the involvement of α -synuclein in the etiology of PD.^(11,12)

Oxidative stress and mitochondrial dysfunction have also been strongly implicated in PD pathogenesis. Oxidative stress results from increased production (or decreased detoxification) of extremely reactive free radicals, including reactive oxygen species (ROS) and peroxynitrite. Free radicals produce oxidative damage by reacting with DNA, lipids and proteins. ROS may be formed during a number of cellular processes, including mitochondrial oxidative respiration and dopamine metabolism. At several sites along the mitochondrial electron transport chain (ETC), there are sites of 'electron leak'. These electrons may combine with molecular oxygen and thereby form ROS, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Of particular relevance to PD, there is a site of electron leak within complex I of the ETC, proximal to the binding site for inhibitors such as rotenone (Fig. 3). Targets of oxidative damage may also include components of the ETC; this may set up a positive feedback loop of ETC inhibition,

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Abbreviations: 3-NT, 3-nitrotyrosine; 6-OHDA, 6-hydroxydopamine; DAT, dopamine transporter; ETC, electron transport chain; GSH, glutathione; H_2O_2 , hydrogen peroxide; L-DOPA, levodopa; MAO, monoamine oxidase; METH, methamphetamine; MPP⁺, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; O_2^- , superoxide; PD, Parkinson's disease; PQ, paraquat; ROS, reactive oxygen species; SN, substantia nigra; SOD, superoxide dismutase

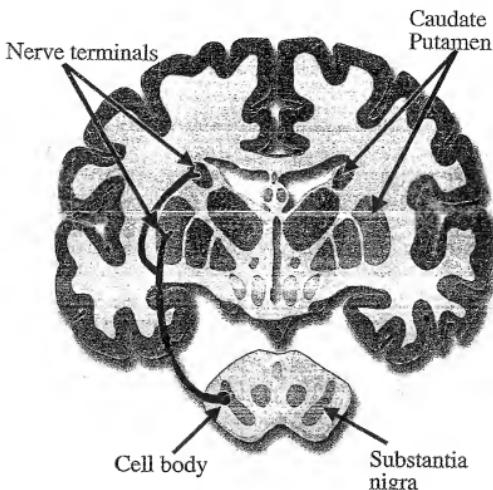


Figure 1. Schematic diagram showing the nigrostriatal dopaminergic pathway, progressive degeneration of which leads to the major symptoms of PD. A cross section of the human brain shows the caudate and the putamen, which constitute the striatum. A section through the midbrain shows the substantia nigra (SN). Dopaminergic neurons (red) whose cell bodies are located in the SN send projections that terminate and release dopamine in the striatum. With degeneration of the dopaminergic pathway, there is progressively less dopamine released in the striatum. Striatal dopamine deficiency, in turn, results in complex changes in the brain's motor circuitry and causes the motor deficits characteristic of PD.

Increased ROS production and further ETC inhibition.⁽¹³⁻¹⁵⁾ In addition, the activities of tyrosine hydroxylase and monoamine oxidase, two enzymes involved in dopamine metabolism, produce H₂O₂ as a normal byproduct. Moreover, auto-oxidation of dopamine results in the formation of ROS,⁽¹⁶⁾ which can participate in a positive feedback loop responsible for progressive oxidative damage.⁽¹⁷⁾ Thus dopaminergic neurons and their nerve terminals, the primary targets in PD, are believed to exist in a constant state of oxidative stress. Cellular free radical scavenging systems, including glutathione (GSH) and superoxide dismutase (SOD), can, to a large extent, prevent ROS from damaging cellular and mitochondrial structures. Partial inhibition of complex I at ETC greatly increases ROS production,⁽¹⁸⁾ which may overwhelm such protective mechanisms. Conversely, depletion of GSH can cause oxidative damage to complex I and a reduction in its activity.⁽¹⁹⁾

Oxidative stress-related changes have been detected in brains of PD patients.⁽¹⁷⁾ These include elevated oxidative damage to DNA, proteins and lipids, decreased levels of

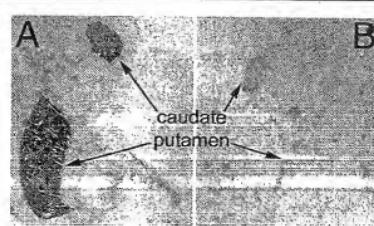


Figure 2. Photomicrographs of human brain sections immunostained for the dopamine transporter (DAT). Antibodies to DAT have been used as an indirect indicator of dopamine innervation of the striatum. Sections are through the striatum and globus pallidus from a person without PD (A) and from a PD patient (B). Note the markedly reduced DAT-immunoreactivity in the PD brain section compared to the control.

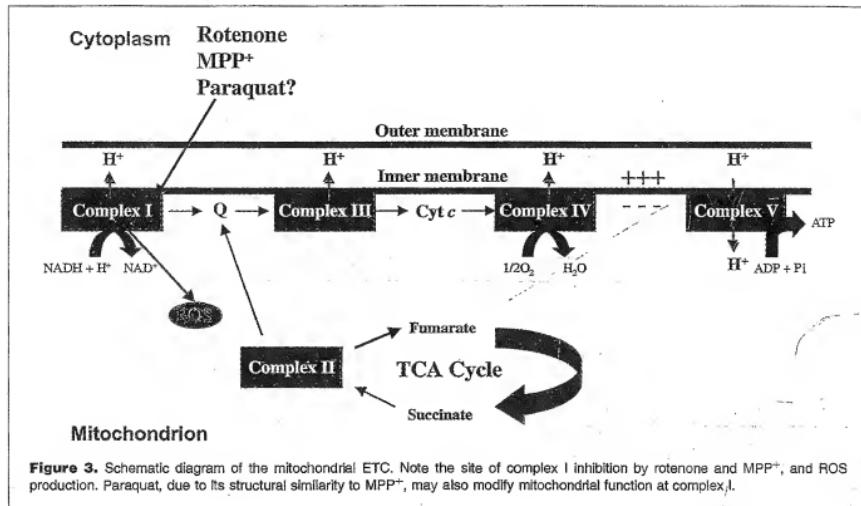


Figure 3. Schematic diagram of the mitochondrial ETC. Note the site of complex I inhibition by rotenone and MPP⁺, and ROS production. Paraquat, due to its structural similarity to MPP⁺, may also modify mitochondrial function at complex I.

reduced glutathione and increased SOD and monoamine oxidase (MAO) activity in PD SN. As suggested above, increased oxidative stress has been attributed to reduced complex I activity of the mitochondrial ETC.^(14,15,16) Of particular interest in this connection, therefore, is mitochondrial dysfunction, which has been suggested to play an important role in PD pathogenesis. Several investigators have demonstrated reduced levels of complex I activity in a variety of tissues from PD patients including brain, platelets, muscle, and fibroblasts.⁽²⁰⁻²²⁾ Furthermore, the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenyl-2,3-dihydropyridinium ion (MPP⁺), has been found to be a mitochondrial toxin that inhibits complex I of the ETC, possibly suggesting complex I involvement in PD pathogenesis.^(23,24)

Despite years of research, however, the mechanisms responsible for chronic, progressive degeneration of nigral dopaminergic neurons remain elusive.

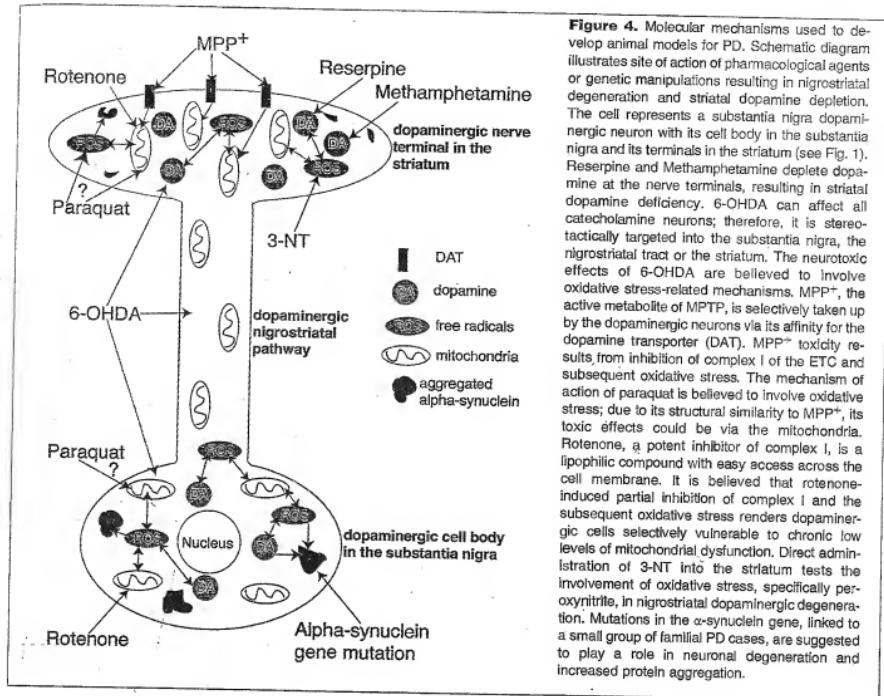
Why use animal models?

Animal models are an important aid to study pathogenic mechanisms and therapeutic strategies in human diseases. Through the use of an animal model, the striatal dopamine deficiency was associated with symptoms of PD, and levodopa (dihydroxyphenylalanine or L-DOPA) was first used to compensate for striatal dopamine loss. L-DOPA therapy still remains the gold standard of treatment of PD.⁽²⁵⁾ Prolonged use of L-DOPA however, commonly results in debilitating,

involuntary movement (dyskinesia), confounding the usefulness of the drug. Moreover, the pathogenesis of PD is not well understood to this day. It is, therefore, of great importance to develop animal models for (a) better understanding the pathogenesis of PD and (b) discovering therapeutics to treat PD.

The present review describes the use of pharmacological agents and environmental toxins to develop experimental PD models (Fig. 4) in a wide variety of species. The aim of these chemical compounds has been to disrupt the nigrostriatal dopaminergic pathway and thereby mimic the striatal dopamine deficiency observed in PD patients. More recently, the discovery of mutations in the α -synuclein gene in a subset of PD patients has led to the development of genetically engineered flies and mice that overexpress the mutant human α -synuclein gene.

As noted, animal models may be useful for studying pathogenic mechanisms, for testing therapeutic strategies, or both. As such, no single model is likely to be suitable for all studies. Animal models are valuable only to the extent to which they accurately simulate the pathogenic, histological, biochemical or clinical features of PD that an investigator wants to examine. This review therefore discusses the advantages and disadvantages of the various animal models of PD and considers their potential roles in revealing the mechanisms responsible for PD pathogenesis, and for testing experimental therapeutics (Table 1).



The Reserpine model

Carlsson et al.,⁽²⁶⁾ observed that systemic administration of reserpine causes depletion of brain catecholamines, leading to an akinetic state in rabbits. Furthermore, they showed that L-DOPA administration alleviated the reserpine-induced akinetic state, indicating that behavioral recovery is dopamine-dependent. This led to the major hypothesis, later confirmed in humans,⁽²⁶⁾ that the motor symptoms of PD result from striatal dopamine depletion.⁽²⁷⁾ The discovery that striatal dopamine deficiency resulted in PD-like symptoms prompted the development of the 'reserpine animal model'. Systemic reserpine administration depletes dopamine at the nerve terminals (Fig. 4) and induces a hypokinetic state in rodents. These movement deficits are due to loss of dopamine storage capacity in intracellular vesicles.⁽²⁸⁾

The principal drawback of this model is that reserpine-induced changes are temporary and striatal reserpine administration does not induce morphologic changes in the

dopaminergic neurons of the substantia nigra. Also, reserpine administration induces the release of other neurotransmitters that may not be directly implicated in PD. Nevertheless, this model has been used successfully to investigate the therapeutic effects of striatal dopamine replacement agents including L-DOPA and dopamine receptor agonists.⁽²⁹⁾ The predictive value of symptomatic drug testing in the reserpine model is imperfect, however, since some drugs that reverse reserpine-induced locomotor deficits are ineffective in PD.

Methamphetamine model

The amphetamines are psychostimulatory drugs with addictive potential. Their activity is primarily associated to their dopamine-releasing mechanism.^(30,31) At very high doses, amphetamine has neurotoxic effects on rodents and non-human primates.⁽³¹⁻³³⁾ Like reserpine, methamphetamine (METH) administration results in dopamine depletion at the level of dopaminergic nerve terminals (striatum; Fig. 4) with

Table 1. Characteristics of animal models of Parkinson's Disease

Model	Symptoms	Histopathology	Pathogenic relevance	Applications	Disadvantages
Reserpine	Akinesia, catalepsy	None	Pharmacological dopamine depletion	Preclinical testing of therapies to improve symptoms	Nonspecific liberation of monoamine transmitters; hypothermia
Methamphetamine	No clear parkinsonian symptoms	At very high doses: loss of TH in striatum; ?loss of dopamine cells in SNc	Dopamine-related oxidative stress	Screen antioxidant therapies to protect dopamine cells	Acute; limited histopathological change
6-OHDA	Unilateral: rotation after apomorphine; Bilateral: akinesia	Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc	Oxidative stress	Preclinical testing of therapies to improve symptoms; screen pharmacological and genetic therapies designed to protect dopamine cells	Acute; usually unilateral (hemiparkinsonian); intrastriatal injection may produce more chronic degeneration; requires intracerebral injection
MPTP	Akinesia, rigidity, tremor in some species	Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc; some loss of locus caeruleus neurons; α -synuclein aggregation	"Environmental" toxin; oxidative stress; inhibition of mitochondrial complex I	Preclinical testing of therapies to improve symptoms; screen pharmacological and genetic therapies designed to protect dopamine cells	Generally acute; non-progressive or reversible; inclusion bodies are rare
Paraquat-Maneb	Decreased locomotor activity	Decreased striatal TH-immunoreactivity	Multiple environmental toxins/pesticide exposure; oxidative stress	Screen pharmacological and genetic therapies designed to protect dopamine cells	Not yet extensively investigated/ described
Rotenone	Akinesia, rigidity, tremor, flexed posture, piloerection	Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc; some loss of locus caeruleus neurons; inclusions reminiscent of Lewy bodies	Chronic "environmental" toxin; chronic oxidative stress; chronic inhibition of mitochondrial complex I	Screen pharmacological and genetic therapies designed to protect dopamine cells	Labor- and time-intensive; substantial morbidity and mortality
3-Nitrotyrosine	Amphetamine-Induced rotation	Decreased striatal TH-immunoreactivity; degeneration of TH-immunoreactive neurons in SNc	Oxidative stress	Screen antioxidant therapies to protect dopamine cells	Not yet extensively investigated/ described; requires intracerebral injection
Transgenic α -Synuclein	Reduced or abnormal motor activity	α -Synuclein-positive intraneuronal inclusions; degeneration of TH-immunoreactive neurons observed in flies; modest decrease in striatal TH-immunoreactivity in mice	Known pathogenic mutations	Screen pharmacological and genetic therapies designed to protect dopamine cells	Expensive and time-consuming; mice do not have characteristic PD pathology or phenotype

minimal effect in the nigral cell bodies.⁽³⁴⁾ However, unlike reserpine, the mechanism of METH action is unclear. Indirect evidence suggests that METH acts through the dopamine receptor and transporter since selective antagonists are able to block its toxicity.⁽³⁵⁻³⁷⁾ Antagonists of the N-methyl-D-

aspartate receptor, such as MK-801, are also capable of inhibiting METH-induced toxicity.^(38,39) The neuroprotective effects of glutamate receptor antagonists on the METH-model may be related to glutamate receptor-regulated dopamine release.⁽⁴⁰⁾ In vitro studies have suggested oxidative stress,

via dopamine autoxidation, and excitotoxicity,⁽⁴¹⁾ as a result of perturbations in energy metabolism,⁽⁴²⁾ as some of the causative factors in the neurotoxic actions of METH.

The major drawback of the METH model is that the histological changes of PD, including degeneration of dopaminergic neurons and presence of intracellular inclusions have not been documented. Furthermore, it is an acute model of striatal dopamine depletion. However, the METH model has been used extensively for biochemical and physiological studies of the dopamine-depleted striatum to better understand such changes in the PD brain.

The 6-OHDA model

6-Hydroxydopamine (6-OHDA) was the first chemical agent discovered that had specific neurotoxic effects on catecholaminergic pathways.^(43,44) 6-OHDA uses the same catecholamine transport system as dopamine and norepinephrine, and produces specific degeneration of catecholaminergic neurons. Systemically administered 6-OHDA is unable to cross the blood-brain barrier. To specifically target the nigrostriatal dopaminergic pathway, 6-OHDA must be injected stereotactically (Fig. 4) into the substantia nigra, the nigrostriatal tract or the striatum.^(45,46) Following 6-OHDA^(46,47) injections into SN or the nigrostriatal tract, dopaminergic neurons start degenerating within 24 hours after, and striatal dopamine is depleted 2 to 3 days later.⁽⁴⁸⁾ The magnitude of the lesion is dependent on the amount of 6-OHDA injected, the site of injection and inherent differences in sensitivity between animal species. Extensive striatal dopamine loss (80–90%) is achieved in most studies and corresponds to specific behavioral changes. When injected into the striatum, 6-OHDA produces a slow, retrograde degeneration of the nigrostriatal system over a period of weeks.⁽⁴⁶⁾

Usually 6-OHDA is injected in one hemisphere while the other hemisphere serves as an internal control. Unilateral injections lead to asymmetric circling motor behavior after administration of dopaminergic drugs, due to physiologic imbalance between the lesioned and the unlesioned striatum. Moreover this turning behavior can be quantified and correlates with degree of lesion.⁽⁴⁴⁾ Although 6-OHDA-induced lesions have been described in mice, cats, dogs, and monkeys, rats are most commonly used because of established stereotactic techniques and relatively low maintenance costs.

There is ample evidence for the involvement of oxidative stress in 6-OHDA-induced neurotoxic effects. It has been reported that 6-OHDA-induced degeneration involves the generation of hydrogen peroxide and hydroxyl radicals in the presence of iron.⁽⁴³⁾ The fact that intranigral injection of iron produces similar neurotoxic effects as 6-OHDA may suggest a role for iron in 6-OHDA-induced degeneration.⁽⁴⁹⁾ In addition, studies have demonstrated that 6-OHDA leads to reduction in GSH and SOD activity⁽⁵⁰⁾ and an increase in malondialdehyde⁽⁵¹⁾ levels in the striatum. It has also been shown that

6-OHDA is toxic to mitochondrial complex I and leads to production of superoxide free radicals.^(14,52) The partial, or even complete, prevention of neurotoxic effects of 6-OHDA and iron by prior administration of iron chelating agents,⁽⁵³⁾ vitamin E^(50,54) and the MAO-B inhibitor, selegiline,⁽⁵⁵⁾ may also be regarded as indirect evidence for the formation of free radicals and involvement of oxidative stress.

The 6-OHDA model does not mimic all the clinical and pathological features characteristic of PD. 6-OHDA does not affect other brain regions, such as locus caeruleus, nor does it result in formation of cytoplasmic inclusions (Levy bodies) like those seen in PD. Furthermore, the acute nature of the experimental model differs from the progressive degeneration of the dopaminergic nigral neurons in PD.

Despite these limitations, the 6-OHDA lesion model has been used to ascertain the efficacy of antiparkinsonian compounds.⁽⁴⁷⁾ Additionally, this experimental model has been useful for evaluating the efficacy of cell transplantation, and for testing neurotrophic factors, compounds that promote survival of the degenerating dopaminergic nigral neurons in PD.⁽⁵⁶⁾

The MPTP model

Inadvertent injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) resulted in clinical symptoms remarkably similar to sporadic PD in humans.⁽⁵⁷⁾ Researchers have capitalized on this discovery to develop an animal model of PD. After administration, MPTP crosses the blood-brain barrier and is metabolized in astrocytes to its active metabolite, 1-methyl-4-phenyl-2,3-dihydroxydiphenylidinium ion (MPP⁺), by monoamine oxidase-B. MPP⁺ is selectively taken up into dopaminergic neurons via its affinity for the dopamine transporter (Fig. 4), and is thus selectively toxic to dopamine neurons.⁽⁵⁸⁾ MPP⁺ toxicity is believed to result from inhibition of complex I of the mitochondrial ETC (Fig. 3) leading to oxidative stress.^(23,24) This mechanism of action of MPP⁺ suggested a role for mitochondrial dysfunction in typical PD. Subsequently, PD patients were found to express systemic reductions in complex I activity.^(20–22)

MPTP administration is one of the most common animal models used to study PD. Exposure to MPTP results in nigrostriatal dopaminergic degeneration in a number of species,^(24,59) including mice, cats, and primates. Susceptibility to MPTP varies across species and strains of animals. For unknown reasons, rats are resistant to MPTP toxicity and mouse strains vary widely in their sensitivity to the toxin. MPTP is usually systemically administered (subcutaneous, intraperitoneal, intravenous or intramuscular). Maintaining bilaterally lesioned animals, especially primates, can be difficult as these animals may need to be maintained on levodopa or other dopaminergic drugs to enable them to eat and drink adequately.⁽⁶⁰⁾ Unilateral intracarotid infusion of MPTP is another method employed in non-human primates wherein the symptoms are expressed mainly on one side, which enables the monkey to maintain

normal nutrition and hydration without medication.⁽⁶¹⁾ Various doses and regimens of MPTP administration (acute versus chronic) are used by different laboratories.⁽⁶²⁾

Acute MPTP exposure results in specific degeneration of the nigrostriatal dopaminergic pathway with 50% to 93% cell loss in the substantia nigra pars compacta and more than 99% loss of dopamine in the striatum.⁽⁶³⁾ However, there is substantial inter-animal variability in terms of effective doses and reversibility of clinical symptoms. In primates, MPTP exposure mimics the behavioral characteristics of PD, including bradykinesia, and rigidity;⁽⁶⁴⁾ however, development of tremor is species-dependent. Neurochemical changes following MPTP exposure include decreased levels of dopamine and its metabolites in the striatum,⁽⁶⁴⁾ increased oxidative damage as evidenced by increased lipid peroxidation,⁽⁶⁵⁾ increased 3-nitrotyrosine levels, and diminished concentrations of antioxidants, such as GSH.⁽⁶⁶⁾ α -Synuclein-positive aggregation in nigral cells of baboons has been observed following MPTP exposure.⁽⁶⁷⁾ The aggregates, however, were unlike the Lewy bodies characteristic of PD pathology.

There are some limitations of the MPTP model of PD. Most protocols of MPTP administration utilize acute drug treatments and fail to mimic the progressive nature of PD. More chronic MPTP treatment may overcome this limitation; however, long-term administration of MPTP, in smaller doses, has resulted in recovery of motor behavior deficits in marmosets once the treatment is stopped. Additionally, the MPTP model does not directly address the involvement of systemic mitochondrial impairment in PD. MPP⁺ inhibits complex I activity solely in cells expressing the dopamine transporter, i.e. dopaminergic cells. Thus, this model only tests the hypothesis that complex I dysfunction, limited to dopaminergic neurons, is toxic to dopaminergic neurons.

The MPTP model of PD has been invaluable in studying the mechanisms of PD pathogenesis. For example, this model suggested a role of mitochondrial dysfunction and environmental exposures in the etiology of PD.⁽⁶⁸⁾ MPTP has also lent support to the oxidative stress model of PD, and has provided clues to the mechanism of cell death in PD. This model has also been useful for testing potential neuroprotective therapies, including drug treatments and dietary alterations, in preventing nigrostriatal dopaminergic degeneration.^(68,69)

Because monkeys have a motor repertoire similar to humans, the clinical features of MPTP-induced parkinsonism are strikingly similar to human PD. As such, MPTP-treated monkeys have provided an important way to test new symptomatic therapeutic strategies, including drugs, transplants, and gene therapy. Currently there is no better, or more predictive model for this purpose.

Paraquat and Maneb

Exposure to the herbicide 1,1-dimethyl-4,4'-bipyridinium, or paraquat (PQ), has emerged as a putative risk factor for PD on

the basis of its structural similarity to MPP⁺, the active metabolite of MPTP. PQ does cross the blood-brain barrier, although slowly, and to a limited extent. When injected systemically into mice, it causes a dose-dependent decrease in dopaminergic nigral neurons and striatal dopaminergic innervation, followed by reduced ambulatory movement.⁽⁷⁰⁾ The mechanism of action of PQ is believed to involve oxidative stress and due to its structural similarity to MPP⁺, its toxic effects could be via the mitochondria (Figs. 3 and 4).

PQ, however, is only one of the many agricultural chemicals known to impact the dopamine systems adversely. Manganese ethylenebisdithiocarbamate, or maneb, which is used in overlapping geographical areas with PQ, has been shown to decrease locomotor activity and potentiate MPTP effects,⁽⁷¹⁾ suggesting that exposures to mixtures of chemicals may also be relevant etiologically. Indeed, combined PQ and Maneb exposures produced greater effects on the dopaminergic system than either of the chemicals alone.⁽⁷²⁾ The paraquat and maneb models give credence to the theory that environmental toxins and pesticides might have a role in PD-pathogenesis. Further investigations using these pesticide models will help to determine the involvement of environmental exposures in the pathological, biochemical, and clinical symptoms of PD.

Rotenone model

The role of environmental toxins and complex I dysfunction in PD is further delineated by a recent study that describes a novel model of PD based on chronic systemic exposure of rats to rotenone, a pesticide and potent inhibitor of complex I.⁽⁷³⁾ A naturally occurring compound, rotenone is commonly used as an "organic" insecticide and to kill nuisance fish in lakes. Additionally, rotenone is a lipophilic compound that easily crosses the blood-brain barrier. Chronic exposure to low doses of rotenone resulted in uniform inhibition of complex I throughout the rat brain. In this way, rotenone exposure differs from that of MPTP, which selectively inhibits complex I in dopaminergic neurons due to its dependence on the dopamine transporter (Fig. 4). Despite this uniform complex I inhibition, rotenone caused selective degeneration of the nigrostriatal dopaminergic pathway (Fig. 5), selective striatal oxidative damage, and formation of ubiquitin- and α -synuclein-positive inclusions in nigral cells, which were similar to the Lewy bodies of PD. Behaviorally, the rotenone-exposed rats were hypokinetic with a flexed posture similar to the stooped posture of PD patients. Some developed severe rigidity and a few had spontaneously shaking paws that were reminiscent of resting tremor in PD.

The rotenone model shows that the features of PD can be produced by systemic complex I inhibition. This indicates that the nigrostriatal pathway is intrinsically and selectively sensitive to complex I dysfunction. The previously described occurrence of complex I dysfunction in PD may further link environmental toxins to the pathogenesis of PD. Many other

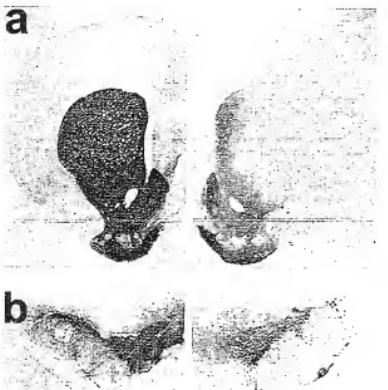


Figure 5. Photomicrographs of rat brain sections after rotenone administration. Sections through the striatum (A) and the substantia nigra (B) stained for tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. Note the markedly reduced TH-immunoreactivity in the rotenone-infused striatum (right) versus the vehicle-infused striatum (left).

environmental agents, in addition to MPTP and rotenone (Fig. 3), affect mitochondrial function at complex I.⁽⁷⁴⁾ Furthermore, complex I impairment may predispose neurons to excitotoxicity and oxidative damage, both of which have been implicated in PD.^(18,75)

The rotenone model appears to be an accurate model in that systemic complex I inhibition results in specific, progressive and chronic degeneration of the nigrostriatal pathway similar to that observed in human Parkinson disease. It also reproduces the neuronal inclusions and oxidative damage seen in PD. Thus, the rotenone model recapitulates most of the mechanisms thought to be important in PD pathogenesis. For this reason, neuroprotective drug treatment trials in this model may be more relevant to PD than other, more acute model systems. The major disadvantages of this model are its labor-intensive nature and its variability, with some animals showing lesions and others not. In addition the sick, bilaterally lesioned animals are difficult to maintain, as with animals treated bilaterally with 6-OHDA or MPTP.

The 3-nitrotyrosine model

A recent animal model was developed to further understand the role of oxidative stress in PD pathogenesis.⁽⁷⁶⁾ As mentioned earlier, oxidative DNA and protein damage and

lipid peroxidation are observed in brains from PD patients.⁽⁷⁷⁾ Antioxidants and free radical spin traps attenuate MPP⁺ toxicity in animal models,^(78,79) suggesting the involvement of free radicals in neuronal degeneration. Recent evidence has implicated peroxynitrite (ONOO⁻) formation in PD pathogenesis. Peroxynitrite is a highly reactive oxidant formed by the reaction of nitric oxide with superoxide anion. Reaction of proteins with peroxynitrite results in the modification of proteins at tyrosine residues (3-nitrotyrosines)⁽⁸⁰⁾ and nitrated α -synuclein is more prone to aggregation than unmodified protein.⁽⁸¹⁾ Furthermore, brains from PD patients show elevated 3-nitrotyrosine,⁽⁸²⁾ suggesting protein nitration may also play a role in the neurodegeneration in PD.

This novel animal model of PD was designed to directly test the involvement of oxidative stress, specifically due to peroxynitrite, in the etiology of PD. Injection of free 3-nitrotyrosine (3-NT) into the striatum (Fig. 4) of mice resulted in loss of striatal tyrosine hydroxylase-positive terminals, loss of dopaminergic neurons in SN, and motor abnormalities.⁽⁷⁸⁾ Thus, these experiments demonstrated that free 3-nitrotyrosine caused neurodegeneration in an animal model. There are some limitations to the 3-nitrotyrosine model of PD, however. Acute exposure to 3-nitrotyrosine fails to mimic the progressive nature of sporadic PD. Additionally, it is still not known whether intrastriatal injection of free 3-NT induces the protein aggregation and cellular inclusions associated with PD. Despite some limitations, this novel model of PD, based on oxidative stress, is useful for understanding mechanisms of PD etiology and has the potential for use in screening putative antioxidant therapies.

Genetic models of Parkinson's disease

The majority of PD cases are sporadic and do not result from obvious genetic defects. A small percentage of patients have a familial form of PD, however, usually marked by earlier disease onset. Mutations in three different genes, including α -synuclein, have been associated with familial PD.^(10,83-85) Since α -synuclein is a major component of Lewy bodies, and mutations in α -synuclein may result in nigrostriatal dopaminergic degeneration (Fig. 4) in familial PD, animal models have been developed to investigate the role of α -synuclein in the etiology of PD. These model systems have focused on the use of transgenic mice or *Drosophila*, which express the wild-type or mutated α -synuclein.

Transgenic mice overexpressing human α -synuclein demonstrate a number of features of PD, including loss of nigrostriatal dopaminergic terminals in the striatum, development of α -synuclein and ubiquitin-positive cytoplasmic inclusions, and motor impairments.⁽¹¹⁾ The inclusions observed in these animals lacked fibrillar organization, which is characteristic of the Lewy bodies observed in PD. Some inclusions were also present in the nucleus, a feature not seen in PD. Dopaminergic and behavioral defects were only observed in

the high expressing line of transgenic mice, suggesting that a critical threshold of α -synuclein expression may be required for the dopaminergic and behavioral abnormalities. Other transgenic mice had α -synuclein-positive inclusions and motor deficits, but there was no evidence of nigrostriatal dopaminergic degeneration. In fact, in these mice, neurons of the brainstem and motor neurons were most vulnerable. Similar pathology was observed in mice expressing wild-type and mutated forms of α -synuclein.⁽⁶⁶⁾

Another interesting genetic approach to studying PD involves expression of normal and mutated α -synuclein in *Drosophila*. These flies demonstrate many features of PD including dopaminergic cell loss, filamentous intraneuronal inclusions and motor defects.⁽¹²⁾ Because of the well-characterized *Drosophila* genetics and the short lifespan of flies, this model offers a valuable opportunity to uncover novel proteins involved in PD pathogenesis. For example, this approach may uncover suppressor genes that prevent dopaminergic degeneration or susceptibility genes that exacerbate the effects of α -synuclein expression.

Animal models based on the transgenic expression of wild-type and mutated α -synuclein provide an important opportunity to study the involvement of α -synuclein in PD pathogenesis. However, there are some limitations to the use of transgenic α -synuclein models. Not all transgenic mouse models demonstrate key features of PD, such as nigrostriatal dopaminergic degeneration. Furthermore, it should be kept in mind that one of the mutations associated with human PD is normally expressed in mice.⁽⁶⁷⁾ Despite these limitations, transgenic α -synuclein mice provide an excellent model system for studying the formation of α -synuclein-positive protein aggregates. This issue warrants detailed investigation since protein aggregation is associated with a number of neurodegenerative disorders. Additionally, these transgenic mice provide accurate models for examining the interplay between genetic mutations and environmental exposures in the etiology of PD. For example, transgenic α -synuclein mice can be used to demonstrate sensitivity to various environmental toxins. Such studies may uncover important contributions of genetic and environmental interactions to PD.

PD has also been associated with parkin and UCH-L1 mutations,^(64,65,68,69) indicating that the ubiquitin and proteasome pathways deserve intensive investigation. Development of good transgenic models will facilitate this line of investigation.

Relevance of the experimental models of PD

Parkinson's disease develops gradually with the typical symptoms becoming fully expressed over a long period of time. A number of animal models of PD have been developed to understand the pathogenesis of this disease, as well as to test potential therapeutics. Each model system has advantages and disadvantages as discussed throughout this review and as summarized in Table 1. Many models of PD use acute toxin

exposure to induce destruction of the nigrostriatal neurons. Although the relevance of these acute models to PD pathogenesis is uncertain, they can be used to screen drugs for symptomatic treatment of the disease. Transgenic models are useful for evaluating the role of genetics in PD. The choice of model to be used depends upon the goals of the particular experimental paradigm and the questions being asked.

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EXHIBIT D

REVIEW

Modeling Parkinson's Disease in Rats: An Evaluation of 6-OHDA Lesions of the Nigrostriatal Pathway

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Human idiopathic Parkinson's disease (PD) is a progressive neurodegenerative disorder that is primarily characterized by degeneration of the dopaminergic neurons of the nigrostriatal pathway. Different 6-OHDA rat models of PD have been developed in which this toxin has been injected into different parts of the nigrostriatal pathway: (a) the medial forebrain bundle which leads to extensive dopamine (DA) depletion; (b) the substantia nigra pars compacta, which leads to more specific and moderate DA depletions; and (c) subregions of the caudate-putamen complex (CPu), which also leads to specific DA depletions. In this article we review the dopaminergic depletion and behavioral consequences of 6-OHDA lesions in the rat. It was examined whether the relation between DA depletion and behavioral deficits mimic idiopathic PD. In addition, it was evaluated which model most closely approximates the human situation, especially in relation to the stage of this progressive disease. It was concluded that with respect to the site of the lesion, rats with partial lesions of the ventrolateral CPu are the most appropriate models to study early and late stages of PD. The choice of the behavioral parameters determines the use of unilateral or bilateral lesions, although it is obvious that the bilateral model mimics the human situation more closely. © 2002 Elsevier Science (USA)

Key Words: 6-hydroxydopamine; rat; animal model; Parkinson's disease; striatum; substantia nigra; medial forebrain bundle; dopamine; behavior; motor function.

INTRODUCTION

Idiopathic Parkinson's disease (PD) is a neurodegenerative disease that affects approximately 1% of the

U.S. population over the age of 55 (6). In 1817 James Parkinson described the motor disorder that now bears his name (46). PD or *paralysis agitans* is a progressive degenerative disorder. The types of symptoms present and their severity depend significantly on the length of time since onset, the rapidity of functional decline, and whether the patient received medication. The movement disturbances can be separated into positive symptoms (behaviors that do not likely occur in healthy people) and negative symptoms (deficits in or loss of a normal behavioral capacity) (35). The positive motor symptoms of PD are a tremor at rest, muscular rigidity, and involuntary movements due to L-DOPA treatment; the negative motor symptoms are bradykinesia (poverty or slowing of movement) and postural disturbances. Apart from the motor deficits patients with PD often exhibit cognitive dysfunction as well. In the most extreme cases, the individual suffers from dementia (a severe impairment of memory, abstract thinking, language, and other cognitive processes). Also, in approximately 40% of PD patients depressive symptomatology has been found (18).

Although PD has an unknown etiology, postmortem studies have established degeneration of nigrostriatal dopamine (DA) as the hallmark of idiopathic PD (34). However, the exact threshold of nigrostriatal DA dysfunction for the clinical expression of parkinsonism is not known. Before symptoms of PD become apparent, 50–60% of the neurons in the substantia nigra (SN) and about 20% of the DA innervation in the putamen can still be found (33). Because of compensatory phenomena, this substantial (approximately 80%) loss of DA levels in the striatum is thought to be necessary before symptoms become obviously manifest (28). Therefore, the diagnosis of PD can only be made when the individual has already had the disease for a certain time. The nigrostriatal system presumably has considerable reserve capacity to endure deficits of over 50% without symptomatic manifestation. Compensatory re-

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sponses by the surviving dopaminergic neurons and also by the postsynaptic cells in the striatum help mitigate the progressive loss of DA innervation. Compensatory responses by afferents to the dendrites of dopaminergic neurons in the substantia nigra (SN) have been reported as well (3). An increased metabolic turnover and hence heightened activity of the remaining dopaminergic cells is one type of compensatory response. A second type of compensatory response is an increased postsynaptic DA receptor density and/or sensitivity. Postmortem brain studies generally indicate modest but significant increases in D_1 and D_5 receptor binding in the putamen of PD patients (59). Based on numerous animal studies, such changes are thought to be a type of denervation supersensitivity. The severe akinesia observed at later stages of the disease is commonly associated with an average loss of neurons in the SN in the range of 60–80%. In addition, DA levels are reduced by over 95% in putamen, but only by 60–90% in the caudate nucleus (8).

The nigrostriatal dopaminergic pathway consists of the A9 cell group, which is located in the substantia nigra pars compacta (SNC). The axons of these neurons run along the medial forebrain bundle (MFB) and terminate in the dorsal striatum. The striatal complex can be divided into a dorsal part (nucleus caudatus and putamen) and a ventral part (including the nucleus accumbens). In the human brain, the nucleus caudatus and the putamen are segregated anatomically by the internal capsule. In contrast, the rat brain lacks such an anatomical segregation and therefore the brain structure is referred to as the caudate-putamen complex (CPu). Because of extensive loss of dopaminergic neurons of the A9 cell group in PD, there is a dramatic decline in striatal DA, leading to the motor impairments. In addition to the SNC, there is also significant degeneration within the midbrain A8 (retrotrubral area projecting to the ventrocaudal putamen) and A10 (ventral tegmental area, VTA, projecting to the nucleus accumbens) dopaminergic cell groups (30). Because of the topographic projections of the A8, A9, and A10 cell groups, the dopaminergic pathway to the putamen is more heavily damaged than the corresponding pathway to the nucleus caudatus and the nucleus accumbens. PD is often thought of as a DA-specific disorder. However, numerous histological studies have demonstrated loss of cells in a number of nondopaminergic cell populations, including noradrenergic neurons of the locus coeruleus (LC) and dorsal vagal nucleus, serotonergic neurons of the dorsal raphe, and cholinergic neurons within the substantia innominata (particularly in the nucleus basalis of Meynert) and in the pedunculopontine nucleus (30, 31). Damage to these important neuronal systems may play a significant role in some of the non-movement-related aspects of PD (e.g., cognition and depression).

THE 6-OHDA RAT MODEL OF PARKINSON'S DISEASE

Experimental models of PD are needed to gain insights into the possible pathological mechanisms of the disease. In addition to this function, they are essential in the development and testing of new therapeutic strategies, whether pharmacological or otherwise. In modeling PD a major advance came with the introduction of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) (27). This molecule is transported into the cell bodies and fibers of both dopaminergic and noradrenergic neurons. It causes degeneration of nerve terminals and can also affect cell bodies, particularly when administered to the cell body regions. 6-OHDA neurotoxicity is based on its potent inhibitory effect on the mitochondrial respiratory enzymes (chain complexes I and IV) (24). Due to metabolic deficits of the blockade of these enzymes, the neurons can no longer exert their normal physiological functions and consequently they die (for a recent overview see Ref. 10). Since in PD it is mainly the dopaminergic nigrostriatal pathway that is subject to degeneration, animal models have been developed in which 6-OHDA lesions of the dopaminergic system were made. Reasonable selectivity for DA is achieved by pretreating the subjects with desimipramine, a noradrenergic transporter blocker that inhibits 6-OHDA uptake into the noradrenergic neurons. Further selectivity for the nigrostriatal tract can be achieved by injecting the toxin directly into distinct parts of this ascending pathway.

In the preclinical research of PD, rat models have been widely used in which 6-OHDA was injected into either one of three target sites. 6-OHDA was injected into the SNC, MFB, or the CPu. It remains obscure, however, which of these models is most appropriate in modeling PD. To model PD the animal model must mimic both the dopaminergic cell loss and the behavioral deficits associated with idiopathic PD. When a model is obtained that meets this standard, insights into the possible pathological mechanisms of the disease might be obtained and neuroactive agents can be tested that might alleviate PD symptomatology.

Injection of 6-OHDA into the MFB

The animal model of PD that to this day undoubtedly has contributed the most in preclinical PD research is the rat with a unilateral 6-OHDA lesion of the MFB (62). Injection of 6-OHDA into the MFB unilaterally can cause a total destruction of A9 and A10 cell groups (48), resulting in the following well-described syndrome: (a) near total depletion of DA in the ipsilateral CPu, (b) denervation supersensitivity of the postsynaptic DA receptors in the ipsilateral CPu, and (c) a characteristic turning behavior in response to both D-amphetamine and apomorphine (see Fig. 1). The completely lesioned (A9 and A10) hemiparkinsonian (i.e., parkinsonianlike syndrome induced in one hemisphere)

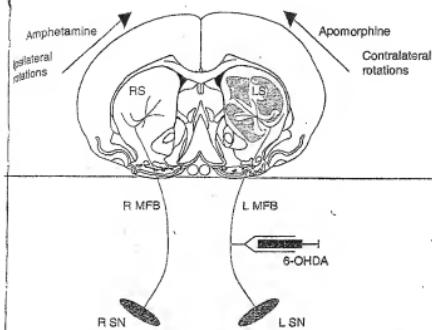


FIG. 1. Diagram of the nigrostriatal pathway and rotational responses produced by apomorphine (contralateral rotations) and α -amphetamine (ipsilateral rotations). Shaded areas in the left striatum indicate the loss of DA due to a MFB injection of 6-OHDA. Abbreviations: RS, right striatum; LS, left striatum; R MFB, right medial forebrain bundle; L MFB, left medial forebrain bundle; 6-OHDA, 6-hydroxydopamine; R SN, right substantia nigra; L SN, left substantia nigra [adapted from (47)].

rat exhibits more extensive neurodegeneration than is seen in human idiopathic PD (see Ref. 48). In contrast, a hemiparkinsonian rat model that is characterized by unilateral destruction of only the nigrostriatal (A9) pathway while leaving the mesolimbic (A10) pathway intact parallels more closely the extent of neurodegeneration seen in human idiopathic PD (48). This was investigated by Perese *et al.* (48) using a selective lesion of the A9 pathway which was almost complete, i.e., tissue DA content in the lesioned CPu was reduced with more than 99%. The selective (A9) versus total (A9 and A10) lesion was achieved by a different location of toxin injection and a different concentration.

Unilaterally lesioning the MFB causes a striking asymmetry in the motor behavior of the rats. Following unilateral MFB lesioning, rats initially tend to turn preferentially toward the side of the lesion, a postural motor asymmetry of behavior that may recover only slightly if the depletion is near total. When challenged with drugs acting on the DA system they will display active rotational behavior (63). An imbalance in DA activity between the two striata causes the rotational asymmetry. Thus, the animal rotates away from the side of greater activity (65). Subcutaneous administration of the DA-releasing agent α -amphetamine creates a DA imbalance that favors the nonlesioned nigrostriatal projection and thus produces ipsilateral rotations (see Fig. 1). This imbalance can be detected even in rats with 50% sparing of nigral dopaminergic neurons, as measured with tyrosine hydroxylase (TH) immunocytochemistry (26). The postsynaptic agonist apomorphine induces rotation contralateral to the lesioned

side (see Fig. 1) because of a stimulation of denervation-induced upregulated D_2 receptors in the denervated striatum (65). Postsynaptic supersensitivity occurs only after most of the dopaminergic neurons in the SNC (approximately 90%) have been eliminated (26). At lower levels of cell loss compensatory increases in DA synthesis by spared dopaminergic cells in the SNC (1) or high levels of endogenous DA in the extracellular space of the denervated CPu (13) could avoid the development of supersensitivity. Rats with more restricted lesions of the SNC show no rotational asymmetry in response to apomorphine (26). Consequently, in many studies the two rotation-inducing agents α -amphetamine and apomorphine have been used to behaviorally assay the extent of neuronal loss following lesions of the SNC (e.g. Ref. 12). It has been demonstrated that rats, which are moderately lesioned (with a 75–90% reduction in dopaminergic fiber density in the CPu), rotated on α -amphetamine but not on apomorphine (29). However, it has to be noted that control rats which received no lesions were often seen to rotate extensively on α -amphetamine. Although it has not been reported, this might be due to a natural imbalance in DA between the two CPu complexes (cf. Ref. 19). However, such an explanation was not discussed (29). More extensive lesions of the CPu (>90% reduction in CPu dopaminergic fiber density) and concomitantly SN (>50% depletion of dopaminergic neurons) are required to generate rotations demonstrable with a low dose of apomorphine but not with α -amphetamine. It was therefore concluded that apomorphine, rather than α -amphetamine, is a better predictor of extensive lesions of the CPu produced by 6-OHDA (29).

It has been shown that a unilateral injection of 6-OHDA into the MFB resulted in rats that either rotated after the administration of apomorphine, Apo (+), or did not rotate after the administration of apomorphine, Apo (−) (6). In the Apo (+) rats a 99.8% depletion of DA tissue content in the CPu and an 85% depletion of DA tissue content in the SN were demonstrated. For the Apo (−) rats the striatal and nigral DA tissue contents were about 75 and 55%, respectively. These results confirm the conclusion that apomorphine-induced rotational behavior is a good indicator of extensive CPu lesions by 6-OHDA. These complete and partial unilateral depletions of the MFB impaired the hierarchic phases of paw reaching differently as measured in a skilled motor task in which animals have to reach for a food reward using fine, controlled movements of the forepaw (staircase test, see Table 1). Barneaud *et al.* (6) reported that a complete DA depletion, but not a partial one, decreased the number of attempts made with the contralateral paw and induced a bias toward the ipsilateral paw, i.e., the ipsilateral paw was preferentially used by these rats as has also been found recently by Dunnett *et al.* (32). A partial DA lesion impaired the sensorimotor coordination of both paws.

TABLE 1

Overview of Several Behavioral Tests with a Brief Description

Procedure as described by Ellenbroek *et al.* (22). This is a test for akinesia. The test apparatus consists of a polyvinylchloride (PVC) frame with four holes. Two holes are for the forelimbs of the rat and the other two are for the hindlimbs. As soon as the rat is placed with all four limbs in the holes the rat is released. The latency to retract the contralateral fore- and hindlimbs from the holes is registered.

Procedure as described by Olsson *et al.* (45). This is a test for akinesia. The rat is held with one hand by the experimenter fixing the hindlimbs (slightly raising the torso) and with the other hand fixing the forelimb that is not to be monitored. In this way the other forepaw has to bear the weight. The rat is moved slowly sideways in both forehand and backhand positions. This is done for both the contralateral and ipsilateral forepaw. The number of adjusting steps for both directions and both paws are counted. Procedure as described by Lindner *et al.* (37). This is a test for rigidity. The experimenter places one hand along the side of the rat and gently pushes the rat laterally. The number of forelimb adjustment steps with the forelimb on the side to which the rat is being moved is counted for both directions.

Procedure as described by Montoya *et al.* (42). This is a test for fine motor control. The test apparatus consists of a clear Perspex chamber with a hinged lid. To this chamber a narrower compartment with a central raised platform running along its length is connected, creating a trough on either side. Due to the narrowness of the side chambers, the rats can use only their left paw for reaching into the left trough and their right paw for the right trough. A removable double staircase is inserted into the end of the box, sliding into the troughs on either side of the central platform. Each of the eight steps of the staircase contains a small well, and two saccharin-flavored pellets are placed in each well. The number of pellets eaten during the test period indicates the rat's success in reach the food and how far the rat can reach. The number of missed pellets remaining at the end of the test on the floor of the side compartment indicates a lack of sensorimotor coordination in grasping and retrieving pellets.

Apomorphine or D-amphetamine-induced rotations
Procedure as described by Ungerstedt (65). This is a test to ascertain maximal lesions. Drug-induced rotations are measured using an automated rotometer consisting of a rotation bowl and a tether attached to the torso of the rat.

Locomotor activity
Procedure as described by Cools *et al.* (15). The rats are placed on a 160 × 160 cm horizontal flat glass table serving as openfield, 95 cm high and surrounded by a neutral white background. Behavior is recorded with a computerized and automated tracking system.

Reaction-time (RT) task
Procedure as described by Amalric *et al.* (2). This is a test for motor initiation. Food-deprived rats are placed in operant boxes, each with a retractable lever and a stimulus light located above the lever. A food pellet magazine is located to the right of the lever. Animals are trained to hold down the lever. After the presentation of the visual cue stimulus at a randomly variable time interval the rats have to release the lever within 700 ms to get a reward (food pellet). The performance of the rats is measured as the number of correct and incorrect (premature lever release or lever release after the 700 ms time interval) trials.

Fixed ratio (FR) bar-pressing task
This is a test for skilled motor control. Food-deprived rats are placed in operant boxes, each with a retractable lever. During the experiment the rats has to make five lever presses [fixed ratio 5 schedule, Cousins *et al.* (16)] or 10 lever presses [fixed ratio 10 schedule, Barnéoud *et al.* (5)] to receive one food pellet as a reward.

Morris water escape test
Procedure as originally described in Morris *et al.* (44) see also (63). A black tank with a diameter of about 1.5 m is filled with water. The rats are placed in the (aversive) water and have to swim toward an escape platform (about 10 cm in diameter). Parameters as swimming distance and swimming velocity are measured. A place version of this test (hidden platform below water surface) measures spatial learning (cognition). In contrast, a cue version in which the platform is visible above the water surface assesses sensorimotor function.

Forelimb use asymmetry test
Procedure as described in Schallert *et al.* (56). The rat is placed in a transparent cylinder. During a time period of 5 min the rearing behavior of the rat is scored. The behavior is analyzed during rearing and landing. The percentage of simultaneous and asymmetric use of the paws during these movements is determined.

Besides impairments in using the contralateral limbs for skilled movements in tests of reaching, impairments in movements of spontaneous food handling have been reported after unilaterally lesioning the MFB (67). Rats with lesions similar as the latter had a

unilateral DA depletion that exceeded 99% in the ipsilateral CPu. It has also been shown that MFB lesions affected place (cognitive) and cue (sensorimotor) orientation in the Morris water escape task (see Table 1) (68). Rats with unilateral MFB lesions that caused

over 95% tissue DA content depletion in the CPu were impaired in the rate of acquisition in both versions of the Morris water task, although they were eventually able to learn both versions, i.e., to locate the escape platform. These results suggest that unilateral destruction of the MFB affects sensorimotor and cognitive skills.

The MFB was also lesioned unilaterally and it was demonstrated that depletion of DA levels in the CPu by over 80% resulted in dramatic reductions in the ability of rats to make adjusting steps (see Table 1), whereas rats with DA level reductions in the CPu of less than 30% showed no detectable deficit (14). Originally, this test was conducted by letting the animal bear weight on a forepaw and monitoring the time until initiation of movement (55). Subsequently, a variety of stepping parameters were studied in rats with a unilateral 6-OHDA lesion of the MFB (45). They reported that adjusting steps were reduced consistently after the unilateral MFB 6-OHDA lesion. In contrast to apomorphine-induced rotations, the deficit in adjusting steps was evident at milder DA depletion, i.e., at 80% reduction in CPu DA levels (14). Furthermore, a depletion of CPu DA levels of about 80% resulted in forelimb use asymmetry in a cylinder (see Table 1) (61). The behavioral deficit was highly correlated with the DA depletion.

Spirduso *et al.* (60) showed that unilateral lesioning of the MFB caused deficits in high-speed motor initiation, comparable to reaction time responding. The movement initiation deficits of the contralateral paw were found to be linearly related to the DA loss, measured as [³H]DA uptake by the CPu. The behavioral deficits in this task were found with a DA depletion of about 40–100%. Thus, this task is sensitive to detect a weak to severe DA depletion. In addition, small yet substantial deficits were also seen in ipsilateral paw performance following more severe lesions, which may be related to DA depletions found in the nonlesioned CPu (60).

When modeling PD with 6-OHDA lesions of the MFB in rats, it is also possible to make bilateral lesions. Using a bilateral model of PD has several advantages. Since idiopathic PD is bilateral, the real pathological situation will be more closely approximated. Another advantage is that sprouting of axons from an intact side of the brain to the contralateral is avoided (66, but see 9). This implies that the model will be more stable with respect to compensation after the lesion. Rats with extensive bilateral MFB lesions were reported to manifest a severe motor symptomatology with akinesia reminiscent of that seen in advanced PD (64). Unfortunately, the extensive bilateral lesion also causes aphagia (deficit in swallowing) and adipsia (deficit in drinking) in the rats (64), requiring them to be tube-fed, which has somewhat limited the use of the bilateral 6-OHDA lesion model. The MFB has also been lesioned bilaterally by the use of a bilateral 6-OHDA

intracerebroventricular injection (68). These rats displayed a tissue DA depletion in the CPu of about 99% and were unable to acquire either the place or the cue version of the Morris water escape task. These findings further indicate cognitive impairments in addition to motor impairments in the MFB lesion model.

Injection of 6-OHDA into the SNC

In order to make a more selective animal model of PD with more moderate DA depletions, the SN has been targeted for 6-OHDA injection. Animals that received a medial and a lateral injection or a single lateral injection into the SNC had moderate sparing of dopaminergic neurons (12). In these rats, the dopaminergic cell loss in the SNC as measured with TH immunocytochemistry was 88%. The percentage of surviving dopaminergic cells in the VTA on the lesioned side was 69% when compared to the unlesioned side. This means that the toxic effects of 6-OHDA are not restrained to the SNC, but that the VTA is affected partially as well. Interestingly, rats that received either a single lateral injection into the rostral SNC or a medial and lateral injection into the rostral SNC had greater sparing of dopaminergic cells in the medial SNC than in the lateral SNC (12, 20). This relative sparing of dopaminergic cells in the medial SNC reflects the pattern of cell loss in brains of patients with PD, in which the DA depletion is mainly lateral (23, 25). Also very interesting is the fact that the number of TH-immunoreactive neurons remaining in the lesioned SNC was correlated with the number of rotations induced by apomorphine, but not with D-amphetamine-induced rotations (12). Consistent with other studies (e.g., Ref. 26) typically only animals in which the lesioned SNC contained 10% or fewer TH-immunoreactive neurons than the intact side rotated after administration of apomorphine. Furthermore, it was demonstrated that TH-immunoreactive fibers in the CPu showed a distribution that paralleled the pattern of nigral cell sparing. The lateral CPu on the side of the lesion was less densely innervated than the lateral CPu (12). In addition, the percentage of CPu area that was innervated by TH-immunoreactive axons was strongly correlated with the number of TH-immunoreactive cells in the SNC. Finally, the percentage of CPu area that was innervated by TH-immunoreactive fibers was also correlated with the number of rotations in response to apomorphine, but not to D-amphetamine. Thus, this animal model with partial lesions of the SNC can predict the extent of cell loss in SNC and axonal loss in the CPu, using the apomorphine-induced rotational behavior as an indication of cell loss.

Since PD affects the brain bilaterally, it is obvious that a bilateral model can be used for preclinical research. Van Oosten and Cools (66) reported about a bilateral 6-OHDA rat model of PD in which the neurotoxin was injected into the SNC. They mentioned sev-

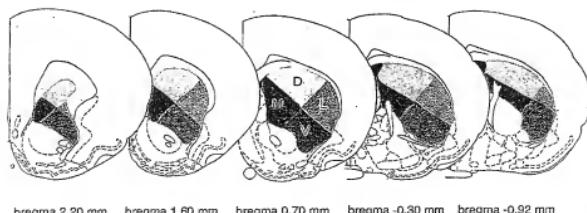


FIG. 2. A rostrocaudal coronal section overview of the left hemisphere of the rat. The gray values indicate the four different parts of the caudate-putamen complex: dorsal, ventral, medial and lateral. The marks under the sections indicate the rostrocaudal position of the coronal section in respect to the bregma (adapted from (47)). Abbreviations: D, dorsal; M, medial; L, lateral; V, ventral.

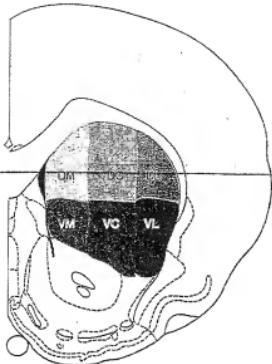
eral rationales for their decision. Among these was the fact that especially the striatal region that is innervated by the nigrostriatal (A9) fibers, and not so much by the mesolimbic (A10) fibers, is depleted in PD. One of the rationales for using a bilateral model was that unilateral injection of 6-OHDA affects the nonlesioned hemisphere as well, which makes a unanimous interpretation of drug-induced effects (e.g., rotational data) difficult. In their study it was shown that relatively small bilateral 6-OHDA lesions, especially of the SNC, and to a minor degree the VTA, produced changes in parameters known to be CPu-specific (66). A unilateral lesion of the SNC was found to deplete DA levels in the CPu by more than 95% on one side of the brain but caused bilateral impairments in skilled paw use, as measured in the staircase test (69). Apparently, unilateral lesions of the SNC do not guarantee behavioral impairments to be restricted to one side of the body.

Injection of 6-OHDA into the CPu

In order to make more selective destructions of the nigrostriatal dopaminergic pathway, the CPu has been targeted as the site of toxin injection in many recent studies. In the majority of these studies discrete subregions in the CPu were selected as a target for the lesion. Terms like ventrolateral and dorsomedial striatum predominate these studies. Using this terminology, there is an important aspect to keep in mind. All the regions named in the following are regions within the dorsal striatum (i.e., the caudate-putamen complex) and not within the ventral striatum (including the nucleus accumbens). Hence, the dorsomedial part of the striatum implies the dorsomedial part of the dorsal striatum or CPu. Figure 2 gives an indication of the regions that are referred to in a rostrocaudal direction. A subdivision is made into a ventral, dorsal, medial, and lateral part. All the other terms are derived from this (e.g., the ventrolateral part of the CPu comprises both the ventral part and the lateral part of the CPu and the dorsomedial part of the CPu comprises both the dorsal and medial part of the CPu).

The ventrolateral sector of the CPu, which receives intensive input from motor and sensorimotor areas of the neocortex and receives its DA innervation exclusively from the SN (33), may be equivalent to the putamen in primates and humans. The dorsomedial CPu, on the other hand, has a mixed DA innervation from both SN (A9 cell group) and VTA (A10 cell group) and receives inputs from frontal cortical areas and the limbic system; therefore this CPu subregion may represent an equivalent of the nucleus caudatus in humans. Lesions involving the dorsomedial parts of the CPu have more general effects on locomotion and drug-induced turning behavior, whereas lesions involving the ventrolateral parts of the CPu have pronounced effects on movement initiation, sensorimotor orientation, and skilled motor behavior (11, 16, 17, 21, 53). The putamen is the striatal subregion that presents the most profound DA depletions in patients with PD (e.g., Ref. 44). Therefore, partial lesions that are focused on the ventrolateral CPu in rats are likely the most relevant models of PD (40).

Kirik *et al.* (33) tried to establish the optimal parameters for a stable unilateral 6-OHDA lesion of the ventrolateral CPu of sufficient magnitude with respect to both the extent of denervation in the CPu and DA cell loss in SN. This was done to induce consistent long-lasting contralateral behavioral deficits in the adjusting steps test (rigidity and akinesia) and the staircase test (fine motor control). They found that this was indeed possible when the neurotoxin was distributed over multiple injection sites along the rostrocaudal axis of the ventrolateral CPu. The functional effects induced by intrastriatal 6-OHDA lesions depended not only on the total dose of the toxin injected, but also on the site of toxin injection. The dose of toxin administered in a single injection had an effect on the extent of CPu DA depletion. When a dose of 6-OHDA was distributed over three or four sites along the rostrocaudal axis of the lateral CPu or when injections were made close to the junction of the globus pallidus, pronounced behavioral deficits occurred. Impairment in



bregma 1.20 mm

FIG. 3. Left rat hemisphere with a CPu subdivision in six areas (Chang *et al.* (14)). The shaded areas indicate CPu subregions. Abbreviations: DM, dorsomedial; DC, dorsocentral; DL, dorsolateral; VM, ventromedial; VC, ventrocentral; VL, ventrolateral [adapted from (47)].

The staircase test was obtained only with a four-site lesion in which an 80–95% reduction in TH-immunoreactive fiber density throughout the rostrocaudal axis of the lateral CPu and 75% loss of TH-immunoreactive neurons in SN. In addition, a 60–70% reduction in TH-immunoreactive fiber density in the lateral CPu, accompanied by a 50–60% reduction in TH-immunoreactive neurons in SN, was shown to be already sufficient for the induction of impairment in initiation of stepping in the adjusting steps test.

Chang *et al.* (14) reported that a reduction in CPu DA levels of 80% or more resulted in impairment in the adjusting steps task. Also, discrete unilateral CPu lesions were used to localize the subregions in the CPu that mediate adjusting steps (14). However, they used a CPu subdivision that differs from the one in Fig. 2 (see Fig. 3). Adjusting steps were shown to be reduced after lesions of dorsolateral, ventrolateral, or ventrocentral CPu (Chang's terminology), but not after lesions of dorsomedial, dorsocentral, or ventromedial CPu (Chang's terminology). Furthermore, none of the discrete CPu lesions resulted in rotation after apomorphine administration, which is consistent with the consideration that apomorphine-induced rotations require the lesions to be maximal.

Bilateral lesions in the CPu would probably serve a closer parallel to the human disease than unilateral lesions because both striata are affected in human PD. Anahric *et al.* (2) investigated the effect of bilateral 6-OHDA CPu lesions on a reaction-time (RT) task which measures motor initiations (see Table 1). In this

RT task the rats are trained to respond to a visual stimulus when holding down a lever. The rats have to release the lever within a certain time interval after the visual cue. An anticipated release of the lever (before the visual stimulus) and a delayed release of the lever (after the predetermined poststimulus interval) are not rewarded by a reinforcer (food pellet). All correct responses are rewarded. The overall decline of DA tissue content in the two lesioned CPu was about 75% of control values. However, lesioned animals differed in the extent of DA depletion, as was demonstrated with TH immunocytochemistry. Thus, the group of lesioned animals could be subdivided into a group with extensive lesions, particularly of the medial CPu, and a group with less extensive lesions that appeared to be restricted to dorsal and lateral parts of the CPu.

Also, animals with asymmetric lesions (one side partly unaffected by 6-OHDA) were observed. These latter lesions had no effect on reaction-time performance. Therefore, it can be concluded that a bilateral DA-depleting lesion has to be symmetrical in both CPu for behavioral deficits to become apparent in this RT task. Animals with the most extensive medial lesions had deficits in both anticipated responses and delayed responses, whereas the animals that were less severely lesioned (dorsally and laterally) showed only an increase in delayed responses (2). Delayed responses and increased anticipated were associated with motor and cognitive deficits, respectively. On basis of these data it was concluded that a large medial CPu DA depletion may provide both motor and cognitive deficits. The dorsolateral CPu is known to receive a dense innervation from cortical sensorimotor areas (41). DA activity in the dorsolateral CPu thus appears to be critical for motor function, i.e., the initiation of movements with temporal constraints. It is mainly the lateral aspect of the CPu that has the emphasis in mediating the function of motor initiation (e.g., Ref. 11). This is confirmed by Cousins *et al.* (16), who reported that mild DA level depletions (about 30% of control levels) in the ventrolateral CPu caused increased initiation times in a skilled motor control task of lever pressing. In this task the rats had to make five presses [in a fixed ratio (FR) five-lever-pressing task] to receive one food pellet as a reward (see Table 1). These motor deficits observed in the rats with DA depletion were argued to show similarities with the motor deficits observed in patients with PD (16).

Besides the motor effects (see also Ref. 52), cognitive deficits could be observed as well after bilateral lesions of the ventrolateral CPu (37). Spatial cognitive deficits, as measured in the (place) Morris water escape task, were reported in two groups of rats with either a 64 or a 60% reduction in nigral neurons. The tissue DA content in the CPu of these two groups of rats was about 55 and 80%, respectively. The differences in depletion could be attributed to age, as the latter rats were middle-aged rats (12 months old), whereas the former

were young adult rats (2 months old). Both age groups were impaired in locating the platform and this cognitive deficit was related to nigrostriatal DA depletion and not to a decrease in dopaminergic transmission in the prefrontal cortex, since DA levels in this latter brain area remained stable after lesioning (37). Furthermore, the cognitive deficits were not likely due to an asymmetry in motor activity, since it has been reported that rats with almost total unilateral CPu DA depletions due to a MFB lesion, eventually learned to locate the platform in the (place) Morris water maze (68).

Besides correlating behavioral deficits to DA depletion in the CPu, it is also important to accurately evaluate compensation following partial dopaminergic lesions. Barnéoud *et al.* (5) recently reported bilaterally lesioned rats (dopaminergic lesion in both the ventrolateral and dorsomedial CPu, which mounted up to a 70% decrease in CPu DA levels) that were able to compensate for some of their behavioral deficits (FR 10 schedule of reinforcement) although initiation of lever pressing after a reward and sustained action were still impaired. This study demonstrated that it is necessary to use several parameters to accurately evaluate compensation following partial dopaminergic lesions (5).

At advanced stages of PD, DA levels are reduced by over 95% in putamen, but by only 60–90% in the nucleus caudatus (34, 44). For investigation in animal models of advanced PD, an animal model providing lasting dopaminergic depletion of 80–100% in the CPu of the rats is needed. Ben *et al.* (7) compared the effects of a single and a double 6-OHDA injection bilaterally into the CPu using the same dose (16 µg/CPu) on DA and 3,4-dihydroxyphenylacetic acid (DOPAC, a DA metabolite) levels in the CPu. Injections of 6-OHDA at two different sites of the CPu (i.e., in the caudal and rostral CPu) induced a relatively stable DA level decrease of about 90% compared with controls. Although the single bilateral 6-OHDA lesioned rats showed a more pronounced loss at week 2 postoperatively than at week 8, which might be indicative of a compensatory phenomenon. This was not observed in double bilateral 6-OHDA lesioned rats. The DOPAC/DA ratio, which might provide a good index of DA release and turnover, was increased in both the single and double bilaterally lesioned groups.

DISCUSSION

Animal models of human idiopathic PD are needed to gain insight into the etiology of the disease itself and to test therapeutic strategies. During the past decades several rat models of PD have been used, but there is a lack of consensus about the location of the lesion, the percentage of DA depletion in the CPu, and the behavioral tests to use to relate the extent of the lesion to the PD-like symptoms. This review shows that the 6-OHDA rat model of PD closely mimics the human

disease. However, as human PD is a progressive disease, it is subdivided into different stages. Subsequent stages of the disease are characterized by a progressive degeneration of the nigrostriatal pathway and a corresponding progressive decline in striatal DA levels. Therefore, to have a reliable and good animal model of PD, an important question has to be met first. In what stage of human PD lies the interest? When PD symptoms start to emerge, about 50% of the dopaminergic neurons in the substantia nigra are lost and striatal DA levels have decreased by about 80% (33, 39). 6-OHDA lesioned rats similar data have been obtained. Compensatory responses to 6-OHDA lesions in the rats have been studied extensively in both unilaterally and bilaterally lesioned animals (13, 70). It was shown that extracellular DA levels in the CPu do not decrease until CPu tissue DA depletion exceeds 80%. At lower levels of DA depletion, neuronal compensation mechanisms are able to compensate for the depletion. These mechanisms involve increased release of DA from remaining dopaminergic terminals as well as an upregulation of DA receptors and supersensitization of DA receptors. Beyond 80% depletion of striatal DA levels, these compensation mechanisms are insufficient in neutralizing the DA depletion. This is confirmed by the finding of a modest and a marked drop in extracellular DA with an 80–95% and >95% depletion of tissue DA concentration in the CPu of rats (13). As a consequence of the inadequate compensatory mechanisms at such high levels of DA depletion, "clinical" symptoms become manifest. Thus, to mimic idiopathic PD, a rat model is needed in which the DA depletion is 80% at least.

In preclinical research, rat models have been developed focusing on the nigrostriatal pathway. This dopaminergic pathway is lesioned at different levels to mimic PD. There is a lack of consensus among researchers about the site for toxin injection. The targets of injection of the neurotoxin 6-OHDA are (a) the site of origin of the nigrostriatal pathway, i.e., the SNC; (b) the axon bundle that partly projects toward the CPu, i.e., the MFB; and (c) the terminal site of the nigrostriatal pathway, i.e., the CPu. In addition, the lesions can be unilateral or bilateral. To mimic idiopathic PD it is important that the DA depletion in the CPu of the rat resembles the situation in the diseased human brain. In addition, the behavioral deficits that can be measured after the lesion should validate the model even more.

Location of 6-OHDA Lesion Site

Medial Forebrain Bundle. The most widely used 6-OHDA rat model for studying PD uses animals with unilateral lesions of the MFB (27). These lesions are almost complete and very few dopaminergic neurons in the SNC survive. This model is valuable as a model of advanced stages of PD. On the other hand, a disadvantage of this model is that not only dopaminergic axons

from the A9 cell group in the SNC are running along the MFB. Axons from the A10 cell group, which terminate in the nucleus accumbens (of the ventral striatum) comprise the MFB as well. Thus, when lesioning the MFB these latter axons are damaged as well. Therefore, MFB lesion models have been developed that are both selective and complete for the nigrostriatal (A9) pathway, i.e., only the A9 cell group is affected and at maximal (about 100%) level (e.g., Ref. 8). However, a less extensive DA depletion after lesioning the MFB has also been reported. Barnéoud *et al.* (6) reported about moderate DA depletions (about 65% CPu tissue DA depletion) after selective MFB lesions. The accompanying mild paw reaching impairments (staircase test) in these animals were proposed as a model of the early symptoms of PD (6). Also, at relatively low levels of DA depletion (a reduction of about 80% in CPu tissue DA content), deficits in the righting steps task (14) and in a reaction time task (60) were observed. In addition to unilateral MFB lesion models, models were developed in which the MFB was lesioned bilaterally. These models have been limited in use, as it appeared that the rats were suffering from adipsia and aphagia (64). Taken together, MFB lesion models mostly have total elimination of dopaminergic cells in the ipsilateral SNC, but also extensive loss of cells in the VTA. Since the extent of this degeneration exceeds the situation in PD, more selective animal models have been developed in which the neurotoxin was injected into either the SNC or into the CPu.

Substantia nigra pars compacta. When using SNC as the target site for toxin injection, PD is mimicked more closely with respect to dopaminergic cell loss. Unilaterally lesioning the SNC was shown to deplete dopaminergic neurons by about 90% in the SNC (12). When compared to MFB lesions [97% loss of TH immunostaining in the SNC (12)], the TH-staining loss in the SNC is somewhat less extensive after SNC lesioning (12). Thus, the dopaminergic cell loss in the SNC model approximates an advanced stage of the human pathological situation. Thus, the pattern of DA depletion was similar to that observed in brains of PD patients, in which the DA depletion in the SNC is mainly lateral (23, 25). Furthermore, in rats that received unilateral 6-OHDA injections into the SNC, the DA depletion in the CPu was also more lateral than medial (12). Individual animals with unilateral DA depletions of 90% or more in the SNC rotated after the administration of apomorphine. In addition to unilateral lesions of the SNC, bilateral models with the SNC as the target site for 6-OHDA injection were used as well. In this model it was shown that small bilateral lesions produced changes in behavioral parameters that are CPu-specific, e.g., via the paw retraction test (see Table 1) (66). A difficulty of the SNC as the target for 6-OHDA injection is the small size of the structure. It

is very difficult to inject the 6-OHDA into this structure without lesioning adjoining structures (e.g., the VTA). This is reflected by the reduction in DA neurons in the VTA by about 30% after SNC lesioning, which was found by Carman *et al.* (12).

Caudate-putamen complex. Partial lesions in the CPu are probably of more value to future preclinical PD research since it can be easily done and the behavioral and biochemical data collected closely approach the human situation. Moreover, rather selective lesions of the CPu can be achieved. But also in modeling PD with CPu 6-OHDA lesions there is no consensus about the lesion site. Some researchers used the dorsomedial CPu (e.g., Ref. 51), whereas others used the ventrolateral CPu (e.g., Ref. 37) and still others used another CPu area for toxin injection (e.g., Ref. 14). Since the putamen represents the most profound DA depletions in the brain of PD patients (e.g., Ref. 44) and the putamen in humans is equivalent to the ventrolateral section of the rat CPu (33), partial lesions aimed at the ventrolateral part of the CPu are probably best. Lesions of this target site showed impairments in behavioral parameters that are associated with PD, like movement initiation, sensorimotor orientation, and skilled motor behavior (11, 16, 17, 21, 53). Furthermore, since PD is a progressive disease, there should be separate models for both early stages of the disease and manifest stages of the disease. The two models are thus dependent on the extent of the lesion and the severity of behavioral deficits.

Partial DA depletion (reduction of 60–80% of CPu DA levels) by local unilateral injection of 6-OHDA in the medial CPu has recently been claimed to be a good model of early and moderate stages of PD in which to examine or study the effects of neurotrophic therapies (e.g., Ref. 36). In this model paw-reaching deficits (staircase test) were observed when CPu DA levels were reduced by about 80%. This is in agreement with a study in which paw-reaching impairments were also obtained with an 80–95% reduction in dopaminergic fibers in the lateral CPu [and a 50–60% reduction in dopaminergic neurons in the SN (33)]. In this latter model, the remaining intact nigrostriatal projection is thought to have a role in the regeneration and functional recovery in response to growth-promoting factors (33). Deficits in paw reaching have been suggested to be similar to the motor deficits seen in patients with PD (16). In one study with a DA depletion of 80% in the CPu rotational behavior after the administration of apomorphine was observed (36). This finding contradicts with most other studies in which apomorphine-induced rotations were only observed in animals with a DA depletion in the CPu of 90% or more (e.g., Ref. 6). The discrepancy of this finding with the other studies can be due to the lesion site, i.e., lesioning the CPu (36) instead of the MFB (6).

Chang *et al.* (14) found that discrete single lesions in

the dorsolateral, ventrolateral, or ventrocentral CPu (Chang's terminology) reduced adjusting steps. The adjusting steps test allows the characterization of non-drug-induced deficits in forepaw movement as a model of akinesia and gait problems, as observed in PD patients (55). Taken together, a discrete lesion in the ventrolateral CPu would be preferable because this part is thought of as equivalent to the putamen in humans. Not only the location of toxin injection, but also the number of injection sites and the concentration of the neurotoxin, is of importance to the model as was shown by Kirik *et al.* (33). Also, the injection volume is important because of differences in diffusion of the neurotoxin from the injection site to the surrounding brain tissue.

Models in which the ventrolateral CPu was lesioned bilaterally mimicked PD closely as well. A depletion of CPu tissue DA content of about 75% induced both motor and cognitive deficits as measured in a RT task (2). The use of a RT task may therefore provide a good index of motor and cognitive abnormalities present in the early stages of PD, as stated by Amalric *et al.* (2). Lindner *et al.* (37) showed that many clinical symptoms could be observed in rats that were bilaterally lesioned in the ventrolateral part of the CPu. In this study, young adult rats had a depletion of nigral neurons of about 65% accompanied with a reduction in CPu DA levels of about 80%. In addition, these rats showed deficits concerning akinesia (FR10 test), rigidity (adjusting steps task), tremor (observation of vacuous or tremulous jaw movements), and cognition (Morris water maze) (37). In the same study, it was attempted to determine whether older rats exhibited more robust parkinsonian deficits than younger rats due to a hypothesized age-related decline in compensatory mechanisms and neuroplasticity. This was hypothesized since the human disease affects people in later stages of their lives. However, the difference between the two age groups was not dramatic. This was probably due to the age of the older rat, which was in fact just middle-aged (12 months), i.e., they were still relatively young to have more CPu damage. Thus, this age is not suitable to test the hypothesis for age-related changes in recovery (38).

Since the dorsoventral and the mediolateral axis of the rat CPu is very important in modeling PD, the question arises whether the rostrocaudal aspect is of importance as well. For instance, it appears that the caudal aspect of the CPu is more damaged by 6-OHDA than its rostral aspect (2, 52). In the studies equated in this review, behavioral deficits in relation to the rostrocaudal axis are not unambiguous. Most of these studies used anterior-posterior (cf. rostrocaudal) coordinates between 1.5 and -1.5 mm from bregma. It remains to be demonstrated, however, whether the behavioral deficits occur beyond this range as well.

Behavioral Aspects of 6-OHDA Lesions

Behavioral deficits in response to 6-OHDA lesions can give an indication of the extent of the lesion. Table 2 gives an overview of models with different target sites for the lesions, the accompanying depletion in the SNC and/or in the CPu, and the behavioral deficits in these models. Models with unilateral lesions often use drug-induced rotational behavior, which can be used as an indicator of nigrostriatal DA depletion. Extensive (maximal) lesions of the CPu (>90% loss of DA fiber density) and concomitantly SN (>50% loss of dopaminergic neurons) are generally assumed to be required to generate rotations demonstrable with low doses of apomorphine, but not with amphetamine (29). Other investigators have argued against drug-induced rotational behavior as a reliable indicator of nigrostriatal DA depletion (e.g., Ref. 14). Chang *et al.* (14) used adjusting steps as an indicator of DA depletion. The stepping test is preferable to acquire an indication of the depletion in DA in experiments in which there is a possibility of damaging the CPu. For instance, grafts can disrupt apomorphine-induced rotation by damaging postsynaptic receptors in the CPu (4). Such damage would further enhance stepping deficits. In contrast, this damage would result in reductions in drug-induced rotation, an effect indistinguishable from any therapeutic effect of substances that are administered to alleviate the symptoms of the disease. Thus, there may be a discrepancy between the interpretation of the behavioral outcome and the actual neuronal damage. The adjusting steps task can also be used as an indicator of submaximal lesions since deficits are detected when DA levels in the CPu are decreased by about 60–80% (14, 37). This fits nicely with the fact that PD symptoms start to occur in the PD patients when DA levels in the CPu decrease beyond 80% (28). Behavioral tests also provide the possibility to assess the extent to which the behavior of the rats relates to clinical symptoms of PD patients (e.g., Ref. 37). Examples of these behavioral tests are the FR-bar-pressing task or the paw-retraction test for akinesia, the Morris water maze for cognitive deficits, the staircase test for fine motor control, and the adjusting steps task for rigidity/akinesia (see Table 1).

Unilateral and Bilateral 6-OHDA Lesions

Most rat models of PD involve unilateral lesions (for an excellent overview of unilateral lesions of the SN or MFB see (57, 58)). These models have been invaluable in preclinical PD research. However, there are several good arguments for using a bilateral model of PD instead. One is that the human disease affects the brain bilaterally as well. Another is that there is no intact site which can partly compensate for the affected site (cf. Ref. 66). In rats with intrastriatal 6-OHDA lesions, it was demonstrated that bilateral 6-OHDA lesions caused impairments in more behavioral motor para-

TABLE 2

Overview of Dopaminergic Lesions in MFB, SNC, or CPu and the Accompanying Behavioral Deficits

6-OHDA lesion site	Concentration 6-OHDA	Percentage reduction in:		Behavioral deficit	Ref.
		SNC neurons	CPu DA levels		
MFB	Unilateral 4 μ g/1.5 μ l	85	99.8	Apo (+): staircase test decrease in use of contralateral paw, bias toward ipsilateral paw	6
	Unilateral 4 μ g/2 μ l	56	72	Apo (-): staircase test deficit in sensorimotor coordination	6
	Unilateral 8 μ g/2 μ l	n.d.	>80	Adjusting steps deficit	14
	Unilateral 8 μ g/2 μ l	n.d.	>95	Apomorphine-induced rotations	14
SNC	Unilateral 8 μ g/4 μ l	n.d.	96	Deficit in (place and cue) Morris water maze	68
	M+L	Unilateral 3 μ g/1.5 μ l + 4 μ g/2 μ l			
	or L	Unilateral 4 μ g/2 μ l	88	n.d.	Only when individual DA depletions were >90% then apomorphine-induced rotations were observed
	n.d.	Bilateral 4 μ g/1 μ l	n.d.	80-100	Deficit in paw retraction test, adjusting steps, and locomotor activity
CPu	C+L	Unilateral 2 \times 3 μ g/2 μ l	27 (SN+VTA)	40	No effect on staircase test or apomorphine-induced rotations
	C+L	Unilateral 2 \times 6 μ g/2 μ l	47 (SN+VTA)	54	No effect on staircase test or apomorphine-induced rotations
	C+L	Unilateral 2 \times 10 μ g/2 μ l	62 (SN+VTA)	82	Staircase test deficit and apomorphine-induced rotations
	L	Unilateral 4 \times 7 μ g/2 μ l	75	80-95 in lateral CPu	Adjusting steps deficit and staircase test deficit
M	Bilateral 12 μ g/3 μ l	n.d.	53 in rostral and 74 in caudal CPu	Motor initiation and response inhibition deficits (RT task)	2
	VC	Unilateral 7.5 μ g/2.5 μ l	n.d.	Adjusting steps deficit	14
	DL	Unilateral 7.5 μ g/2.5 μ l	n.d.	Adjusting steps deficit	14
	DL	Bilateral 12 μ g/3 μ l	n.d.	Motor initiation deficits (RT task)	2
V	VL	Unilateral 7.5 μ g/2.5 μ l	n.d.	Adjusting steps deficit	14
	VL	Bilateral 12.5 μ g/2.5 μ l	n.d.	Motor initiation deficit in FR5	16
	VL	Bilateral 12.5 μ g/2.5 μ l	60-64	Deficit in FR10, adjusting steps, and (place) Morris water maze	37
	C	Unilateral 4 \times 5 μ g/2 μ l	80	20-40 in rostral and 60-70 in caudal CPu	No effect on apomorphine-induced rotations, locomotor activity, and staircase test. Deficit in adjusting steps
C	Bilateral 4 \times 5 μ g/2 μ l	80	20-40 in rostral and 60-70 in caudal CPu	No effect on apomorphine-induced rotations. Deficit in locomotor activity, staircase test, and adjusting steps	52

Abbreviations: 6-OHDA, 6-hydroxydopamine; DA, dopamine; MFB, medial forebrain bundle; SNC, substantia nigra pars compacta; Apo (+), rats rotating after apomorphine administration; Apo (-), rats not rotating after apomorphine administration; RT, reaction time; FR, fixed ratio; M, medial; L, lateral; D, dorsal; V, ventral; C, central; n.d., not determined; CPu, caudate putamen complex; SN, substantia nigra; VTA, ventral tegmental area.

signs than unilateral 6-OHDA lesions (52). Thus, a bilateral model would be preferable in regard to compensatory mechanisms. In a rat model of PD compensation during the experiment complicates the interpretation of the data and therefore it is necessary to control for functional recovery or compensation after the lesion (see "Compensation" below). In models with bilateral lesions, compensation during an experiment

can occur as well, but it is excluded that this is mainly due to sprouting of axons from the other side of the brain. There are, however, some limitations. An obvious one is that in a bilateral model administration of drugs acting on the dopaminergic neurotransmission will not lead to rotational behavior since there is no DA imbalance between the two brain sides. In addition to the lack of rotational behavior, the forelimb use asym-

metry test cannot be used in the bilateral model. However, the bilateral model offers the possibility to assess higher cognitive tasks such as a choice reaction-time task. Hence, the choice of using a bilateral rat model of PD or a unilateral model depends on the aim of the experiment and researchers should be aware of the advantages and shortcomings of the model of interest.

Compensation

Compensation or recovery of functions can be achieved via regeneration of dopaminergic projections from remaining dopaminergic tissue. For instance, it has been shown that unilaterally lesioning the SNC led to a sprouting of dopaminergic fibers in the ventrolateral part of the CPu (9). In the other areas of the CPu, the TH immunostaining of fibers on the lesioned side seemed to be the comparable to the control side. The sprouting of dopaminergic fibers in the ventrolateral CPu was only observed 4 and 7 months after lesioning, but not after 10 days. Since it appeared that the TH-immunoreactive fibers in the ventrolateral CPu were less numerous at 4 months than at 7 months after lesioning, it was suggested that there exists an ongoing process of regrowth of dopaminergic fibers in this part of the CPu up to 7 months (9). This regrowth of dopaminergic fibers is likely to be a compensatory response to a diminished nigrostriatal DA innervation. Assuming a homogenous dopaminergic innervation of the CPu by the nigrostriatal pathway, these latter data would also indicate a differential regrowth of DA fibers in CPu subregions. Moreover, besides compensation that is mediated by sprouting from remaining intact DA fibers of the lesioned side, compensation on the behavioral level can also be mediated by sprouting from DA fibers from the ipsilateral nonlesioned side. This is supported by the recent finding that bilaterally lesioning of the CPu caused additional behavioral deficits besides those observed after unilaterally lesioning the CPu (52). The emergence of these additional behavioral deficits can be explained by the fact that a bilateral lesion reduces the possibility of compensation by sprouting from the intact brain side.

Barnéoud *et al.* (5) reported recovery of function in a fixed-ratio-bar-pressing task after a partial dopaminergic lesion (70% reduction in CPu DA levels). This recovery of function did not apply to all behavioral parameters studied. This recovery of function is possibly due to compensatory mechanisms like sprouting of remaining dopaminergic fibers. Since different behavioral functions are mediated by different parts of the CPu, the recovery of function of some behavioral parameters in time might be due to its mediation via the part of the CPu that is subject to sprouting. It should be noted, however, that other compensatory responses (e.g., elevated DA biosynthesis, metabolism, and release by the remaining dopaminergic neurons) could also contribute to the recovery of function. It has, for

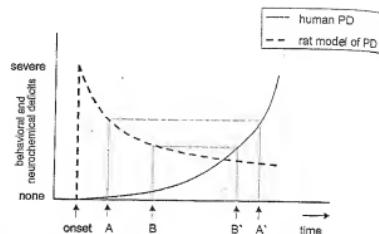


FIG. 4. Schematic overview of course of severity of behavioral and neurochemical deficits in time for both the rat model and human idiopathic PD. Time point A in the rat model of PD is equivalent to time point A' in human PD and time point B in the rat model is equivalent to time point B' in human PD. The arrow marked "onset" indicates the onset of PD symptoms.

instance, been reported that normalization of extracellular DA levels seems to be sufficient to account for recovery of function (49, 50). This was suggested since the time course of increases in extracellular DA levels and behavioral recovery were similar, in contrast to those for DA biosynthesis, metabolism, and DA release (49). In addition to various compensatory responses, even other brain structures (outside the CPu) can be involved in the functional recovery of some of the behavioral parameters. Thus, it should be noted that regenerative processes occur in the 6-OHDA model whenever the lesion is not complete (9).

When inducing a DA lesion in rats, the rats will go from a state of having no parkinsonian symptoms (before the lesion) to a state of displaying severe parkinsonian symptoms. From this moment on compensatory mechanisms will come into action to antagonize the neurobiological deficits. This means that PD symptoms in the rat will be alleviated to some extent in the course of time. In contrast, human idiopathic PD is a progressive disease with a clear reverse development: PD symptoms will worsen in the course of time. This contradiction between the idiopathic situation and the situation in the animal model is illustrated in Fig. 4. It is important to keep in mind that when behaviorally testing the lesioned rat at time point A, time point A' in the human disease is mimicked (see Fig. 4). In addition, it is also relevant to notice that not all behavioral parameters are subject to compensatory mechanisms as was shown by Barnéoud *et al.* (5).

Variation Within the 6-OHDA Model

A further important point to note is that when 6-OHDA is injected into the MFB, SNC or CPu, there will always be some variability among the lesioned animals. This was shown by several investigators (e.g., Refs. 2, 6, 60). Although the target of toxin injection is exactly the same among the animals, the regional dis-

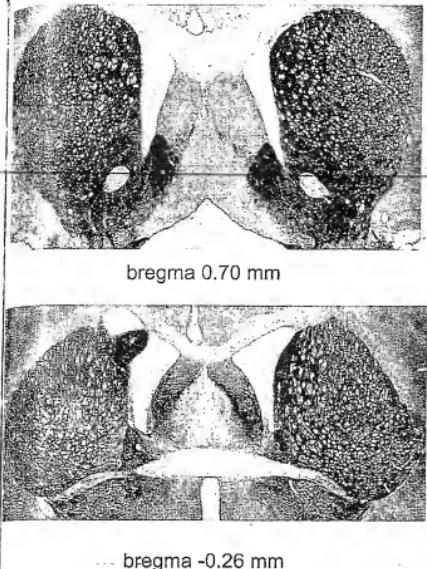


FIG. 5. Two cross sections of a rat brain that was bilaterally lesioned in the CPu. The two sides of the brain show different densities in TH-immunoreactivity staining. This means that although the lesion was aimed to be symmetrical between the two brain sides, the DA depletion differed.

tribution of TH immunoreactivity in the CPu will vary among the rats. This seems to be a drawback of the 6-OHDA model of PD. Furthermore, lesions do not only vary among animals in regard to the extent of the lesion, but in the bilateral model the lateral distribution of TH immunocytochemistry is subject to variation as well (2). Figure 5 shows two cross sections of a rat brain that was bilaterally lesioned in the CPu (own observations). Lesions in both CPu were placed at the same coordinates on both sides of the brain. The bilateral injections ($2 \times 10 \mu\text{g}/2 \mu\text{l}$ per side) were in between the dorsal and ventral CPu (see Fig. 2: crossing point of ventral and dorsal CPu). As can be seen, there is an obvious difference in TH immunoreactivity in the CPu between the two brain sides. Therefore, in order to acquire a good model of PD, the experimental rat population should not be too small. Larger rat populations allow to dissociate groups with different percentages of decline and/or regional differences in DA immunoreactivity. Moreover, a variation in the data allows correlation analysis, especially between anatomical and behavioral data. The results about possible relationships

can be used to gain insight into the functioning of the nigrostriatal pathway during pathological conditions.

Conclusions

A rat model in which the ventrolateral CPu is lesioned bilaterally is probably one of the most suitable models of PD, since MFB lesions are too extensive and since the small SNC limits the use as a lesion site for routine use due to practical difficulties. Unilateral models can be used in preclinical PD research as well. When using a unilateral model of PD and using drug-induced rotations to assess the extent of the lesion, it is important to keep in mind that compensatory sprouting of collaterals from the intact contralateral side, as well as the affected ipsilateral side, of the brain will interfere with the obtained rotational data. Furthermore, human PD is bilateral and therefore a bilateral model is preferable when the objective is to mimic PD more closely. In general, the 6-OHDA rat model of PD mimics the human disease with respect to behavioral and neurochemical parameters. However, an essential difference between the 6-OHDA model of PD and human idiopathic PD is the opposite development of PD symptoms. Human PD has a progressive nature, whereas the 6-OHDA model is subject to compensatory mechanisms. Therefore, different rat models should be used for different stages of human PD. The behavioral deficits can be measured using different behavioral paradigms. For example, the paw retraction test for akinesia, the adjusting steps task for rigidity, the Morris water escape task for cognitive functions, the RT task for motor initiation, and the staircase test for fine motor control.

In conclusion, independent of the site of injection, the 6-OHDA-induced DA depletion appears to be a valuable model to investigate PD symptomatology and to gain more insight into the possible pathological mechanisms of this neurodegenerative disease. However, the choice of using a bilateral model or a unilateral model depends on the aim of the experiment and one should be aware of the consequences of the choice for a certain 6-OHDA model.

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2001-2004 McKnight Brain and Memory Disorders Award
2002 Ameritec Award For Spinal Injury Research
2005 Senator Jacob Javits Award in the Neurosciences

Teaching activity:

Section organizer and lecturer for Clinical Neuroscience Module,
Mechanisms of Disease, Second Year Medical School Curriculum
Course organizer and lecturer, Neuroscience 507/Neurology 108,
Cellular and Molecular Mechanisms of Neurologic Disease
Conference leader, Neurobiology 500b,
"Pathophysiology of Neurodegenerative Diseases"
Attending Physician, Yale-New Haven Hospital
Neurology Inpatient Service
Attending Physician, Yale-New Haven Hospital
Neurology Consult Service
Attending Physician, Resident Firm Clinic
Lecturer, Yale Neurology Medical Student Clerkship
Member, Yale Neuroscience Thesis Committees

GRANT SUPPORT

"Axonal growth cone signal transduction"
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.
Agency: National Institute of Neurological Disorders and Stroke
Type: R37 (NS 33020, Years 13-19) Period: July 1, 2005 to June 30, 2012
This project explores signal transduction in axonal growth cones of cultured neurons, and is focused entirely on the mechanism of semaphorin action.

"Molecular mechanisms of axonal regeneration"
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.
Agency: National Institute of Neurological Disorders and Stroke
Type: R01 (NS/HD 39962 Years 6-9) Period: April 1, 2005 to March 28, 2009
This project deals generally with the determinants of axonal regeneration in the adult animal. The work is focused on Nogo-A, CSPG, SPRR1A and Fn14.

"Nogo Receptor in Axonal Regeneration"
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.
Agency: National Institute of Neurological Disorders and Stroke
Type: R01 (NS 42304, Years 7-10) Period: July 1, 2007 to June 30, 2011
This project seeks to determine the molecular mechanisms of Nogo Receptor action and to determine the role of the Nogo Receptor in the success or failure of adult CNS axon regeneration after injury.

"NogoReceptor Antagonist for Ischemic Stroke Recovery"
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.
Agency: National Institute of Neurological Disorders and Stroke
Type: R01 (NS 082529, Years 1-5) Period: Feb 1, 2006 to January 31, 2011
This project deals generally with the methods to promote recovery from ischemic stroke through axonal plasticity.

Axonal Regeneration Pharmaceuticals for Recovery from Spinal Cord Injury
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.
Agency: Wings for Life Foundation

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